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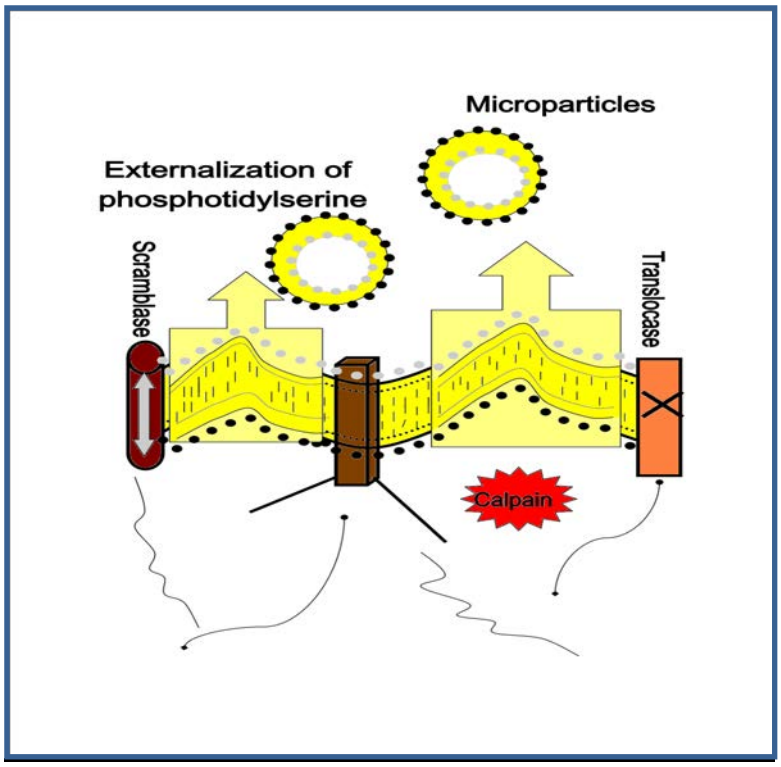

Title: Investigating the role of Microparticles/ Microvesicles/Extracellular vesicles in vascular biology, haemostasis and haemopoietic dysregulation

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This thesis is submitted for fulfilment of requirements for the Degree of Doctor of Philosophy
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Contents

Note : the publications are embedded as pdf and retain their original page numbers as in the manuscript. Each publication has a prelude consisting of the relevant aim, the citation and the key learning points.

PREFACE

Statement of originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository subject to the provisions of the Copyright Act 1968 and any approved embargo.

Thesis by publication

Acknowledgement of Authorship

I hereby certify that the work embodied in this thesis contains published papers of which I am a joint **first** author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty of Health Assistant Dean (Research Training), attesting to my contribution to the joint publications.

Note: I am submitting the PhD thesis by publication. The contents of the literature review and introductory material for each chapter are thus adapted from publications, included in this thesis.

Anoop K Enjeti

Newcastle

Acknowledgements

I am grateful to Dr. Michael Seldon and Dr. Lisa Lincz for their guidance and supervision of this project. I thank all the scientists and laboratory staff of the Hunter Haematology Research Unit for allowing me to use their facilities and for helping me at various stages of the project.

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I thank my colleagues in my department for their support during the course of the project. Finally, I thank my family for their constant support, patience and valuable time throughout my PhD.

Anoop K Enjeti

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Publications included in this thesis

Refereed publications (seven)

PAPER 1: Enjeti AK, Ariyarajah A, D'Crus A, Seldon M, Lincz LF. Correlative analysis of nanoparticle tracking, flow cytometric and functional measurements for circulating microvesicles in normal subjects. *Thromb Res*. 2016;145:18-23.

PAPER 2: Enjeti AK, Ariyarajah A, Warwick E, Seldon M, Lincz LF.Challenges in Analysis of Circulating Extracellular Vesicles in Human Plasma Using Nanotracking and Tunable Resistive Pulse Sensing. *J Nanomed Nanotechnol*. 2017; 8: 468.

PAPER 3: Enjeti AK, Ariyarajah A, D'Crus A, Seldon M, Lincz LF. Circulating microvesicle number, function and small RNA content vary with age, gender, smoking status, lipid and hormone profiles. *Thromb Res*. 2017;156:65-72.

PAPER 4: (Book Chapter) Enjeti AK, Seldon M. Microparticles : Role in Haemostasis and Venous Thromboembolism (ed 2012): InTech; 2012

PAPER 5: Enjeti AK, Lincz LF, Scorgie FE, Seldon M. Circulating microparticles are elevated in carriers of factor V Leiden. *Thromb Res*. 2010;126(3):250-253.

PAPER 6: Enjeti AK, Lincz LF, Seldon M, Isbister GK. Circulating microvesicles in snakebite patients with microangiopathy. Res Pract Thromb Haemost 2019;3:121-5.

PAPER 7: Enjeti AK, Ariyarajah A, D'Crus A, Riveros C, Seldon M, Lincz LF. Circulating microvesicles are less procoagulant and carry different miRNA cargo in myelodysplasia. Blood Cells Mol Dis 2019;74:37-43.

Refereed Appendix publications (three)

Note: These manuscripts were published, with PhD candidate as first author, 3 years prior to commencement of the PhD at the university of Newcastle.

Appendix PAPER 1(Review): Enjeti AK, Lincz LF, Seldon M. Microparticles in health and disease. *Semin Thromb Hemost*. 2008;34(7):683-691.

Appendix PAPER 2(Review): Enjeti AK, Lincz LF, Seldon M. Detection and measurement of microparticles: an evolving research tool for vascular biology. *Semin Thromb Hemost*. 2007;33(8):771-779.

Appendix PAPER 3: Enjeti AK, Lincz L, Seldon M. BioMaleimide as a Generic Stain for Detection and Quantitation of Microparticles. *International journal of Laboratory Haematology*. 2007; Jul 2(3):196-199.

Title page Figure legend

This picture was conceived by the authors and published on the cover page of the 'Seminars in Thrombosis and Haemostasis' journal. It shows the budding of MP (or MV) from the cell membrane by externalization of phosphatidylserine by the interplay of the enzymes scramblase and translocase.

Adapted from Appendix PAPER 1 (Review): Enjeti AK, Lincz LF, Seldon M. Microparticles in health and disease. *Semin Thromb Hemost*. 2008;34(7):683-691.

Abbreviations

APTT Activated Partial Thromboplastin Time

ELISA Enzyme linked immunoabsorbent assay

EV Extracellular vesicles

ISEV International society for extracellular vesicles

ISTH International Society for Thrombosis and Haemostasis

MAHA Microangiopathic haemolytic anaemia

MDS Myelodysplasia

MP Microparticles

MV Microvesicles

NTA Nanotracking analysis

TRPS Tunable resistive pulse sensing

VICC Venom induced consumptive coagulopathy

XaCT Factor X-activated clotting time

CHAPTER 1

SYNOPSIS/OVERVIEW

"As the common Size of the Natives is somewhat under six Inches high, so there is an exact Proportion in all other Animals, as well as Plants and Trees: For instance, the tallest Horses and Oxen are between four and five Inches in height, the Sheep an Inch and a half, more or less: their Geese about the bigness of a Sparrow, and so the several Gradations downwards till you come to the smallest, which, to my sight, were almost invisible; but Nature had adapted the Eyes of the Lilliputians to all Objects proper for their view: They see with great exactness, but at no great distance. And to show the sharpness of their Sight towards Objects that are near, I have been much pleased with observing a Cook pulling a Lark, which was not so large as a common Fly; and a young Girl threading an invisible Needle with invisible Silk."

Jonathan Swift, Gulliver travels

1.1 Summary

Microvesicles (MV) are membrane bound cell-derived particles < 1μm in size and their role in various pathophysiological processes is an area of intense research. MV in circulation are usually derived from blood cells or other tissues in close proximity to the blood vessels. Particles of any size in circulating plasma are now referred to collectively as extracellular vesicles (EV) – these include MV and larger than 1μm vesicles. The role of these MV/EV is diverse and experimental data suggests their role is important to normal as well as abnormal haemostasis and thrombosis. They have now been described to carry and transfer genetic material such as microRNA(miRNA) and regulate distant cell and tissue function. Certain sections of the literature still use the term microparticles (MP) for cell derived particles in circulation. As some journals have used MP in preference, the terms (MV or MV/EV or MP,

where appropriate) are used interchangeably in this work which focusses on the MP/MV compartment of extracellular vesicles.

There are a number of techniques for investigation of MV including flow cytometry, functional clot based assays and ELISA. However, there is limited information on how the techniques compare with one another and practical concerns for translational research with each technique. Moreover, innovative techniques such as tunable resistive pulse sensing (TRPS) and nanotracking analysis (NTA) had been compared only in a limited fashion for complex and heterogeneous samples such as human plasma. Before any meaningful investigation could proceed, it was necessary to understand the relative strengths and weaknesses as well as the relationships between the techniques. This first part of the research project was undertaken after a thorough literature review.

There are a number pathophysiological conditions where the role of MV is important, particularly in haemostasis and coagulation. However, the literature review undertaken also revealed the lack of comprehensive data from normal healthy subjects. We therefore felt it was critical to evaluate the MV distribution in normal healthy subjects before pathological disease cohorts were studied.

The next step was to identify and quantify MV in specific diseases groups where MV could play a role in pathogenesis. Specifically, these were clinical cohorts identified as those with pathological thrombosis (factor V Leiden carriers), pathological bleeding (snake bite victims) and those with a clonal bone marrow disorder (myelodysplasia). They were from patient groups readily accessible at the Calvary Mater Newcastle hospital, a regional centre with expertise in haematology (including thrombosis, bleeding, blood cancers) and toxicology. This included a cohort of factor V Leiden subjects (with and without thrombosis) – an inherited mutation associated with increased risk of thrombosis. Following this, a unique cohort of snake bite patients with venom induced coagulopathy and microangiopathic

hemolysis was evaluated. Finally, we investigated a clonal bone marrow disorder characterised by abnormal blood counts, transfusion dependence and myelodysplasia (with some patients being red cell and platelet transfusion dependent).

In the first group with factor V Leiden (FVL) mutations, both heterozygote and homozygote, we were interested in analysing the association of MV with clinical thrombosis as this condition has a widely variable thrombotic phenotype, particularly in heterozygotes. In the second cohort of snake bite victims with venom induced coagulopathy selected from the Australian snake bite project, we investigated flow cytometric based detection of MV in envenomation. It was postulated that MV could be generated due to toxic injury to the endovascular system. The relationship of MV to venom induced coagulopathy with microangiopathic hemolysis is unexplored.

Myelodysplasia (MDS) is a clonal bone marrow disorder with ineffective haemopoiesis resulting in cytopaenias as well as risk of progression to leukemia with a clinical presentation that includes infections, bleeding and transfusion dependence. Microvesicles detected in relation to cancer have been labelled ‘oncosomes’ and have been described in several tumours including melanoma and prostate cancer. However, there was little information on the role of MV in primary haematological disorders apart from a few studies in myeloproliferative disorders. We were specifically interested in investigating if the type, level, function and miRNA content of MV correlated with clinical phenotype in myelodysplasia. As miRNA are implicated in transfer of regulatory material across cells, we were also interested in interrogating any association with haematopoiesis and angiogenesis in MDS.

1.2 Hypothesis

We hypothesised that the detection/enumeration method is key in determining MV number and function. A combination of different techniques would be important to evaluate various

facets of MV. We also hypothesized that levels and function of MV vary with age, gender, blood counts, lipid/hormone profile and smoking status. Further, the levels and/or function of MV in certain pathological states (such as factor V Leiden and snake bites) influence thrombosis and/or bleeding. Finally, levels and functional haemostatic role of MV and its small RNA cargo are important in clonal bone marrow disorders such as Myelodysplasia and could be associated with clinical phenotype.

1.3 Aims

1. To evaluate different techniques of microvesicle detection and measurement by correlating MV analysis by flow cytometry, functional ELISA, XaCT test, thrombin generation and nanotracking in a cohort of normal healthy subjects.
2. To undertake a comprehensive analysis of variables that affect circulating MV in normal healthy subjects, specifically focussing on age, gender, lipid /hormone profile and smoking status.
3. To undertake a comprehensive analysis of circulating MV in homozygote and heterozygote carriers of Factor V Leiden and compare with normal healthy subjects.
4. To evaluate of levels of circulating MV in a cohort of snake bite patients with venom induced consumptive coagulopathy and microangiopathic hemolyic anaemia.
5. To analyse the levels of microvesicles in a cohort of myelodysplasia subjects by

a) both numerical and functional tests and correlate with disease risk scores, blood counts and
b) to isolate and characterise the small RNA and specifically, miRNA profile of circulating MV in myelodysplasia patients

1.4 Overview of chapters

Chapters two to six cover different aspects of the research project as outlined above. Each chapter has a descriptive section followed by the specific aim and the publication (or manuscript) that addresses the specific aims. Chapter 2 covers introductory concepts on MV/EV and haemostasis, mainly intended for a reader who may be less familiar with this field.

Chapter 3 extensively covers the various techniques for characterising MV/EV; numerically, for cell of origin, and functionally. In the first half of this chapter, the role of the various techniques in MV, including the advantages and disadvantages of each approach is investigated. The findings confirmed that various techniques operated in different size ranges, whilst flow cytometry was particularly useful in identifying the cell of origin. The second half of the chapter evaluated the innovative techniques of nanotracking analysis (NTA) and tunable resistive pulse sensing (TRPS). The results of this part of the study showed that it was important to be aware of the size range within which these techniques operate and there may be specific situations where each of techniques has an advantage.

This is followed by Chapter 4, where the variability of circulating MV/EV in normal healthy subjects, due to differences in age, gender, blood counts, smoking status, hormone and lipid profile is discussed. The key findings included raised MV derived functional procoagulant activity in females and lower MV subsets as well as activity in smokers.

Chapter 5 explores three specific disease cohorts- those with pathological thrombosis (factor V Leiden carriers), pathological bleeding (snake bite victims) and those with a clonal bone marrow disorder (myelodysplasia). In first cohort of subjects with factor V Leiden mutations, it was observed that circulating MV levels were elevated in carriers of FVL but not associated with thrombosis. The second cohort was the snake bite victims with venom induced coagulopathy selected from the Australian snake bite project. It was observed that

red cell MV were markedly increased and a surprising finding of decreased endothelial MV in those with microangiopathic hemolysis. Finally, we also investigated the role of MV in myelodysplasia (MDS), measuring levels, association with disease specific risk factors, and then evaluating small RNA, with a focus on miRNA, isolated from circulating MV. It was observed that procoagulant functional activity was decreased whilst overall small RNA non-coding cargo was significantly increased when compared to age similar normal controls. Specific miRNA species isolated from the MV were also differentially expressed in MDS .

The concluding chapter six summarises the results, challenges faced during the project work and significance of the work, including future directions.

CHAPTER 2

INTRODUCTION AND BACKGROUND

2.1 Introduction to Microvesicles

Microvesicles are small membrane bound vesicles usually measuring 100-1000nm which are shed from a cell surface.[1,2] These are released from activated or apoptotic cells. After long being considered cell dust, MV have been shown to reflect in vitro cell stimulation and occur in vivo under a variety of pathophysiologic conditions. They are now regarded as vectors of transcellular exchange of biologic information. All circulating blood cells including endothelial cells release membranous fragments ~100nm in size or smaller bearing at least some characteristics of the parent cell. Several factors cause production of MV - activation, complement mediated lysis, shearing stress, oxidative injury and active vesiculation.[3] They differ from exosomes (30-100nm) which originate through the exocytosis of endocytic multi-vesicular bodies and play a role in antigen presentation. [4]

The Internal Society for Thrombosis and Haemostasis (ISTH) vascular biology subcommittee developed the following definition for microvesicles/ microparticles[5] :
The generally agreed upon size range is between 100 to 1000nm. However, the lower size limit is contentious with the developments such as atomic force microscopy and nano-scale technologies resulting in improved detection of smaller particles.[6] The vesicles must also:

1. Lack a nucleus or synthetic capacity
2. Be enclosed by a membrane cytoskeleton
3. May arise from a variety of cells and contain a variable amount of surface phosphatidylserine

The above definition must be recognised as somewhat arbitrary and the size criteria reflect the limit of detection for most membrane bound particles at least by one popular method for detection, i.e. flow cytometry. In reality, MV represent a spectrum of different sized particles ranging from very small (less than 100nm) to fragments as large as platelets [as shown in figure 2(a)]. In fact, shed membrane bound particles have long been noted by anatomical pathologists in their specimens from lymph nodes or tumour tissue. In fine needle aspirates from lymph nodes, cytoplasmic fragments shed from lymphocytes known as lymphoglandular bodies, have been observed.[7] Thanatosomes, which are eosinophilic hyaline globules noted on tissue sections in solid tumour have also been described.[8] It is likely both lymphoglandular bodies and thanatosomes are produced by similar pathophysiological mechanisms to microvesicles (such as activation or apoptosis) but are substantially larger, probably several microns in size, in comparison to 'conventional' microvesicles.

This definition of MV or EV should also be viewed in the context of innovative sizing technology such as nanoparticle tracking methods, impedance flow cytometry and spectroscopic techniques. The current understanding is that MV. represent a heterogeneous population of 100-1000nm sized vesicles which actively shed from cell surface and retain characteristics of the parent cell.[9] The more recent MISEV 2014 criteria define the broader EV as the the vesicles found in extracellular fluid or from cultured medium or other body fluids obtained after minimal mechanical disruption of cells and tissues. It is also acknowledged that there is no one definition that is universally accepted or employed. [10]

The term MP or MV, usually refers to vesicles in the size range of 100-1000nm or 0.1-1μm but excludes exosomes. For the purposes of this thesis, the term microparticles (MP) and microvesicles (MV) are used interchangeably. It is noted that most publications prior to 2014 used the term microparticles whilst subsequent publications have used the term microvesicles whilst describing similar experimental work. [1]

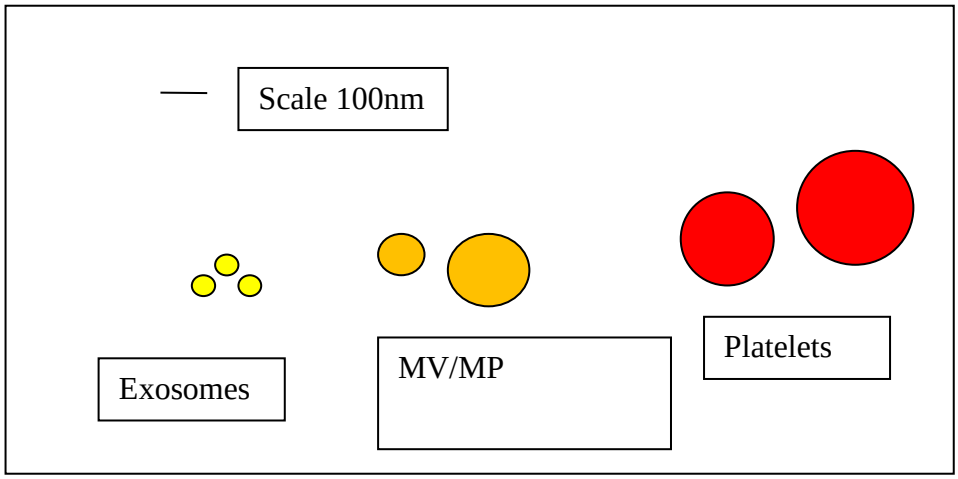

Figure 2(a). Relative sizes of exosomes, microvesicles and platelets. Reproduced and adapted in colour from reference (Enjeti et al, appendix paper 2).[11]

2.2 Extracellular vesicles

Particles of any size in circulating plasma are now referred to collectively as extracellular vesicles. This includes MV, exosomes and any larger than 1000nm vesicles as well.[12] The current widely accepted definitions for extracellular vesicles has 3 broad groups based on size range, sedimentation properties and homogeneity of the vesicles: 1) apoptotic bodies 2) cellular microparticles or microvesicles 3) exosomes as shown in figure 2(b). Further sub-classifications of EVs, such as oncosomes being vesicles that are seen in association with cancer has been proposed, but lack of widely accepted markers make this challenging to achieve. [13,14]

It is now widely accepted that the generic term EV is restricted to extracellular vesicles where their vesicular nature as per surface or functional characterisation is demonstrable. The nomenclature now increasingly includes EV with additional specification such as size and density and /or subcellular origin (apopototic bodies, microparticle, microvesicle and exosome). [15]

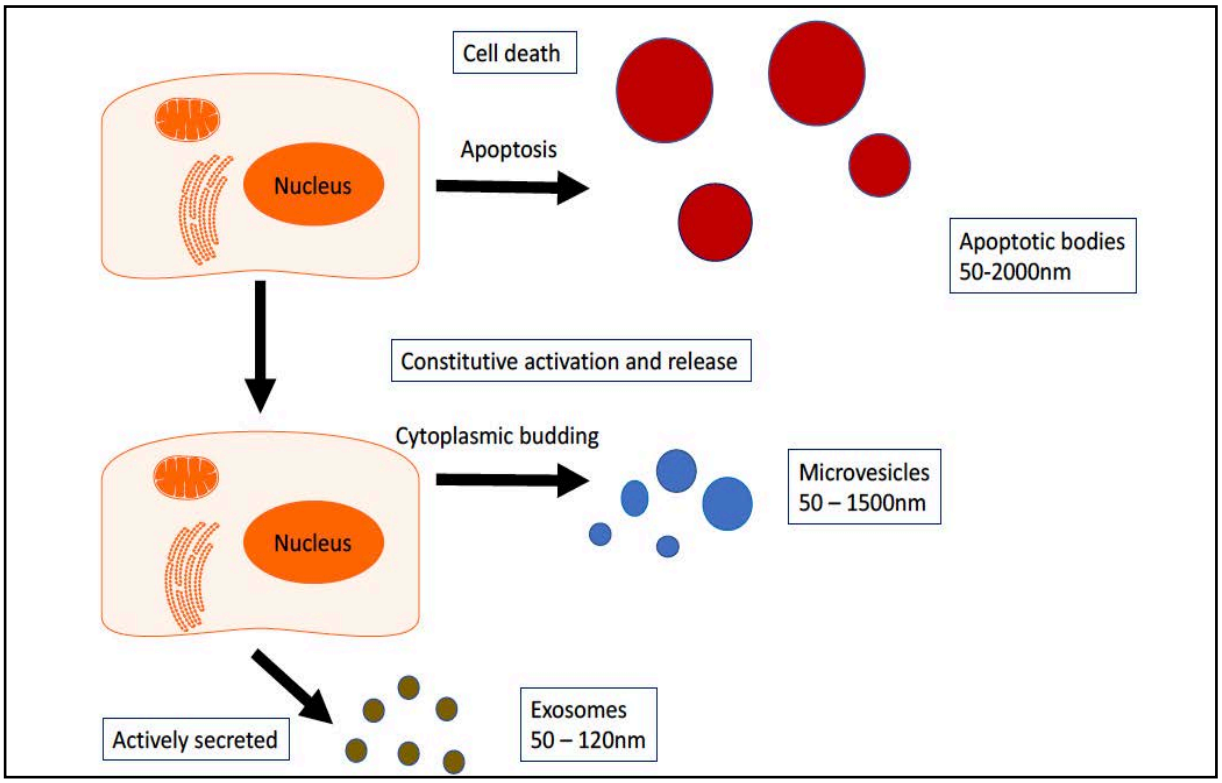

Figure 2(b). Production of apoptotic bodies, microvesicles and exosomes. Adapted from reference. [14]

2.3 Classification of Extracellular vesicles

EV could be classified in different ways based on their inherent properties. An outline of the various categories of extracellular vesicles (including MV) is shown in Table 2(i). The multifaceted EV/MV with immense pathophysiological potential is often considered a 'miniature envoy with many faces'.[16] Based on their potential function and effect, MV with either physiological or pathophysiological roles are described.[1] Elevated levels of MV production may lead to a pathological state, for example, excessive MV from platelets may contribute to thrombosis.

Table 2(i). Classification of Extracellular Vesicles. Adapted from reference (Enjeti et al, appendix paper 1). [1]

Based on biogenesis	**Based on size**	**Based on coagulant property**	**Based on cell of origin**	**Based on function**	**Based on Protein content**
Exosomes Microvesicles Apoptotic bodies	Small (50-120nm) Intermediate (~200-300nm) Large (>500nm)	Anti-coagulant Pro-coagulant	Platelet Endothelial Leukocyte Monocyte Cancer cell	Messenger Adhesion Coagulant Infectious Cell signalling Antigen presentation Transcriptional regulation	Transmembrane Cytosolic Intracellular Extracellular

2.4 Mechanisms of vesiculation

The production of MV occurs by vesiculation or blebbing of the cell membrane. The mechanisms governing the remodeling of the plasma membrane are complex, involving cytoskeletal changes and a shift from normal phospholipid asymmetry. It is generally agreed that sustained increase in intracellular calcium is necessary to allow transmembrane redistribution of phosphatidylserine (PS) to the cell surface.[17] After stimulation, calcium is released from intracellular stores resulting in activation of cysteine proteases, gelsolin and calpain, and subsequent cell membrane instability.[5,18,19] Several enzymes including translocase, floppase and scramblase are actively involved in amino-phospholipid distribution and stability on the membrane surface as seen in figure 2(c)*A*.[18] The function of

translocase is to specifically move phospholipids from the external leaflet to the inner leaflet whereas scramblase bi-directionally moves all phospholipids [see figure 2(c)*B*].[20] The raised intracellular calcium cleaves and destabilizes actin filaments, whilst it activates scramblase and inactivates translocase. As illustrated in figure 2(c)*C*, this leads to cytoskeletal instability due to disruption of the protein anchorage as well as PS reorganization which leads to MV formation.

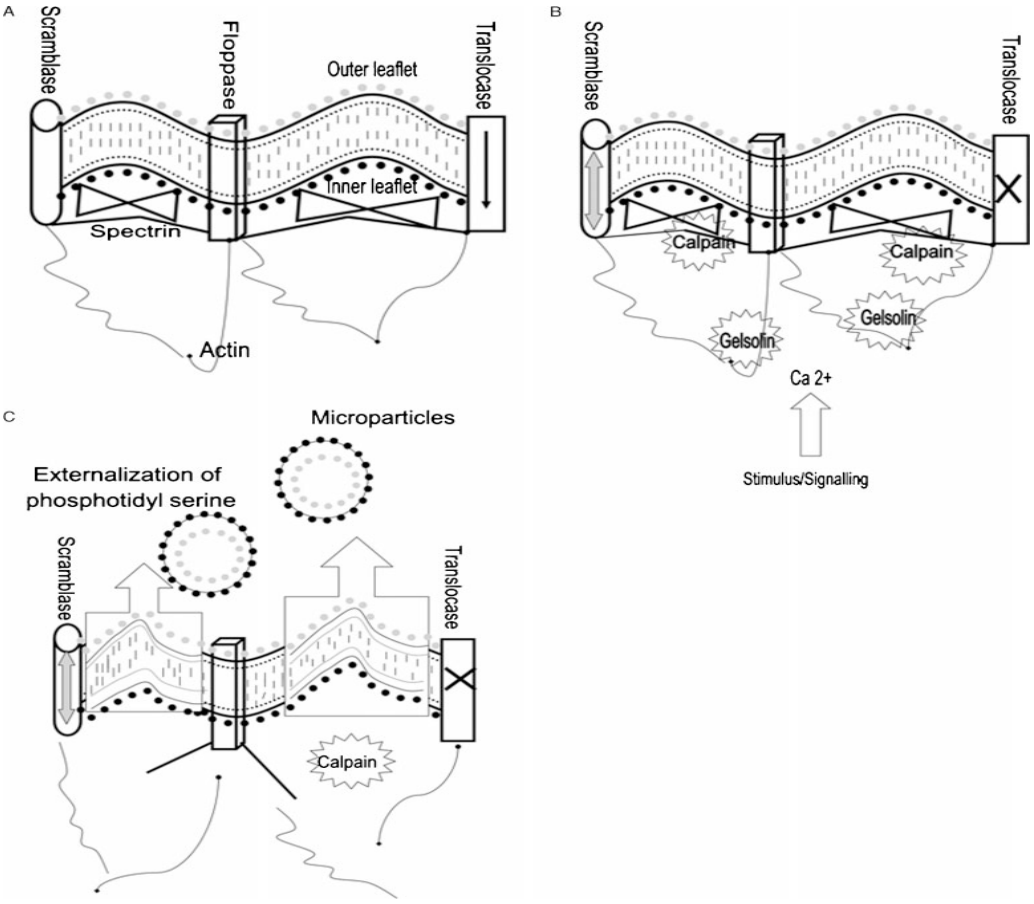

Figure 2(c). (A) Normal membrane asymmetry is maintained by the activity of translocase. This can be disrupted by a variety of stimuli that increase intracellular calcium, which in turn activates gelsolin, calpain, and scramblase while inactivating translocase. (*B*) Gelsolin and calpain cleave actin filaments, and scramblase activity promotes membrane asymmetry. (*C*) This leads to externalization of phosphatidylserine and formation of microparticles. Reproduced from reference (Enjeti et al, appendix paper 2).[17]

2.5 Evolving concepts in understanding of vesiculation

The exact mechanisms of biogenesis and release of MV is still being understood and unfolded. The traditional understanding of vesiculation has been described above. Focal changes in phospholipid and protein componenets of the plasma membrane leading to unequal distribution and subsequent changes in curvature leads to MV budding. This is facilitated by aminoacid translocases (flippases and floppases).

It is generally agreed that the MV formation as a result of plasma membrane budding is a distinct process from the release of exosomes which occurs from the endosomal compartment. However, there is now evidence that shows that the key endosomal machinery is also used to generate MV.[21] Similarly, proteins related to viral budding and other endosomal proteins which can affect plasma membrane curvature have also been implicated. The shift in localisation of these proteins to the plasma membrane allows changes in the membrane that facililate budding.[22] Both cargo sorting and MV shedding are thought to be tightly regulated by several small GTPases from the ARF, Rab and Rho family.[23]

Crowding of proteins and lateral pressure from protein-protein interactions is another recently described possible mechanism for membrane vesiculation.[24] In some specific situations such as vesiculation from red blood cells metabolic processes such as ATP content and redox state could play an important role.[25]

2.5.1 Cytokine release

The cell to cell communication via cytokine release from EV is an emerging concept. A recent publication shows that the association of cytokines with EV varies with the biological system and changes with type of activation.[26] Moreover, specialized assays are required to detect these vesicle encapsulated cytokines. [26]

2.6 Microvesicles in vascular biology and haemostasis

The tissue factor (TF) based model for thrombosis suggests an active role for MV and P-selectin in thrombus formation.[27] TF is expressed in the subendothelial membrane and when damage to the vessel wall occurs a significant amount of TF is released into the bloodstream in the form of microvesicles.[28] This exposure of TF triggers the coagulation system and initiates the formation of a clot. Activated platelets bearing P-selectin are attracted to the site of injury and serve to further recruit MV to the thrombus by increased thrombin generation at the site of injury.[29] Most MV are thought to be procoagulant and increased levels could signify a thrombotic risk. The MV also provide an extensive phospholipid membrane surface for propagation of the coagulation cascade. The interplay of TF MV, endothelial MV and platelet MV and their role in haemostasis is illustrated in figure 2(d)*i* & 2(d)*ii*. The P-selectin bearing platelet MV is said to form an important part of the coagulation triad with tissue factor and the vessel wall, as seen in figure 2(e).[30]

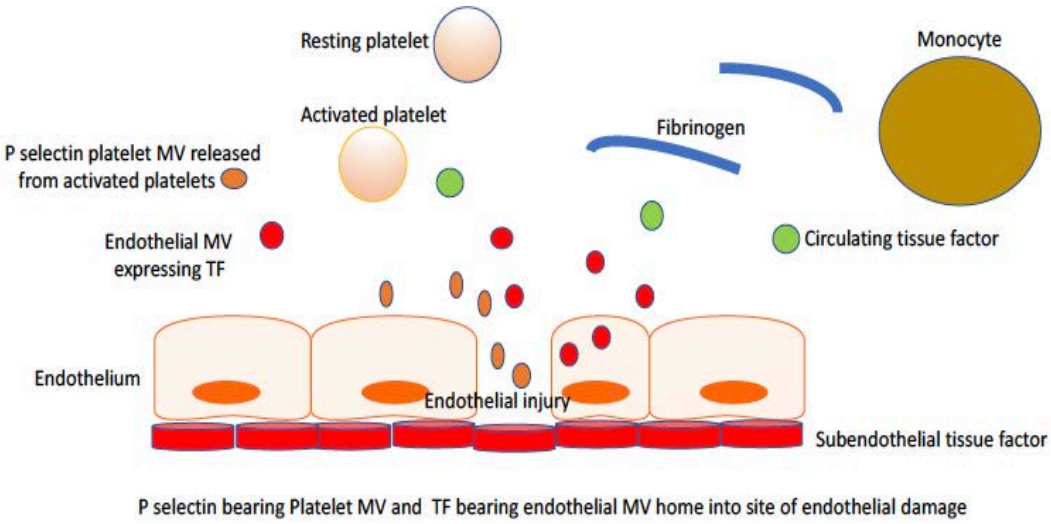

Figure 2(d)*i*. Detailed illustration and model of tissue factor (TF) and P-selectin (from platelet MP) action in thrombus formation. significant amount of TF is released after vessel

wall injury or stimulation of endothelial cells; subsequently, platelet derived p-selectin expressing MV participate in thrombus propagation. Adapted from reference.[30]

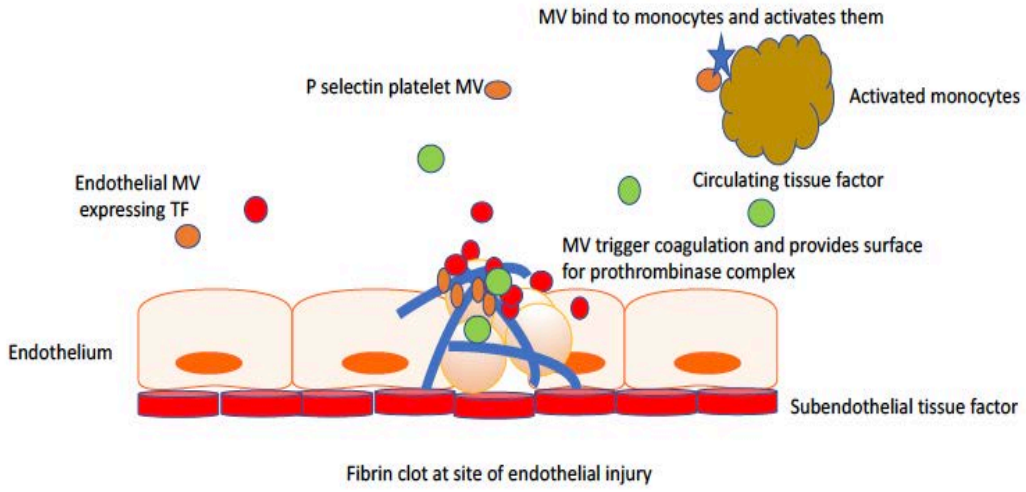

Figure 2*(d)ii*. P-selectin (P) is present on activated platelets and endothelial cells. The P-selectin shed from these cells as MV provides a surface for prothrombinase complex formation and activates monocytes which induces TF-positive MV generation, further propagating the thrombus.Adapted from reference. [30]

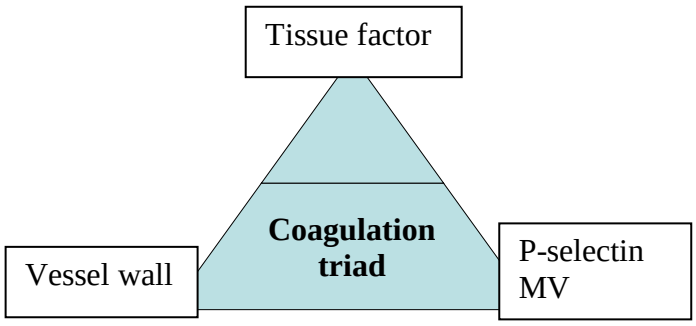

Figure 2(e). Coagulation triad of TF, vessel wall and p-selectin MV.

The role of MV in haemostasis is a complex interaction of endovascular cell derived MV with platelets, leukocytes, fibrin polymerization and other proteins at the site of endothelial injury. Traditional models of haemostasis have focussed on formation of initial platelet thrombus

formed by activated platelets at the site of injury. This is thought to be followed by activation of a number of serine proteases (coagulation proteins) such as factor VII, VIII and IX. The activated coagulation factors sequentially form two complexes which assemble on phospholipid surfaces – the tenase complex (FVIIa/FIXa, with the 'a' indicating the activated coagulation factor) and the prothrombinase complex (FVa/FXa). The latter complex in the presence of calcium, converts prothrombin to thrombin which results in the final step of conversion of fibrinogen to fibrin resulting in a fibrin clot. The MV phospholipid surface provides a vast surface area on which tenase/prothrombinase complexes are able to be formed. The entire process is tightly regulated by a counter regulatory system composed of proteins such as protein C, protein S and anti-thrombin.

The role of tissue factor bearing MV homing in to the site of endothelial injury was recently evaluated in 'in vivo' mouse models and this provides proof of principle for the model described in the above section.[31] Furie's group elegantly demonstrated using intra-vital wide-field microscopy in a laser injury mouse model that platelets, fibrin and tissue factor being MV engage at the site of injury to initiate and propagate the thrombus.[29] They used multicolour fluorescent markers to tag platelets, tissue factor and fibrin. Following the initial release of tissue factor and fibrinogen, tissue factor expressed on MV shed from the injured endothelial wall activates platelets and initiates thrombosis. Their proposed experimental model for thrombosis suggests that P-selectin expressing platelet derived MV and TF expressing MV from leukocytes and endothelial cells rapidly accumulate in the thrombosis upon laser induced endothelial injury.[32,33] This increased concentration of tissue factor leads to further propagation of the thrombus. The platelet thrombus as well as fibrin clot formation significantly overlaps in time, in contrast to the classical model where the platelet thrombus is thought to precede the fibrin clot.

Recent publications in certain cancers have shown that MV, also known as 'oncosomes', have certain unique features. For example, in prostate cancer they have been described to

be atypically large ranging from 1-10μm. [34] Such 'oncosomes' are reported to carry distinct cargo and represent a separate functional class compared to usual MV.[35] Cell to cell horizontal transfer of miRNA mediating diverse processes such as transfer of resistance in cancer cell lines is also a key role.[36-39]

2.7 Other physiological roles for MV

Several other roles include enzyme and messenger functions. In contrast to the discussion in the previous section, some MV have been observed to also have anticoagulant properties as well such as MV with protein C activity. Recent reports of MV being involved in immunological pathways for T cell regulation suggest their importance in autoimmune phenomenon and/or in immune dysregulation.[40]

Appendix PAPER 1 (Review):

Enjeti AK, Lincz LF, Seldon M. Microparticles in health and disease. *Semin Thromb Hemost*. 2008;34(7):683-691.

Learning points

Circulating MV are important role players in normal haemostasis. The role of MV in the pathophysiology of the different disorders is under intense investigation. MV levels are raised in several pathophysiological conditions such as inflammation, thrombosis and cancer. This review was carried out before the period of the PhD.

Note: Due to the accepted manuscript being included in final thesis for Open Access purposes, the page numbers in the thesis are not sequential.

Title: Microparticles in health and disease

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ABSTRACT

Microparticles (MP) are small fragments of membrane-bound cytoplasm that are shed from the surface of an activated or apoptotic cell. Recently, their function as vectors of transcellular exchange of biologic information, in addition to more well described forms of intercellular communication such as growth factors, cytokines and chemokines, has become well recognized. Circulating levels of MP are thought to reflect a balance between cell stimulation, proliferation and death. The production of MP is thought to predominantly occur by vesiculation or blebbing of the cell membrane. The mechanisms governing the remodeling of the plasma membrane are complex, involving cytoskeletal changes and a shift from normal phospholipid asymmetry. Increased intracellular calcium subsequent to cell activation leads to intracellular increases in several proteins including Gelsolin and Calpain, as well as the activity of enzymes such as translocase, floppase and scramblase which play important roles in the homeostasis of the cell membrane. The membrane vesiculation and phospholipids asymmetry leading to the production of MP occurs by the complex interplay of the proteins involved. There are a number of clinical conditions associated with elevated MP - most are also associated with an increased risk of thrombosis. Apart from cardiovascular disease and venous thromboembolism, MP are also elevated in solid tumors with metastatic disease. The measurement of MP is being regarded as a potential biomarker, given the range of conditions in which it is elevated and the association with prothrombotic states. The utility of measuring MP as a diagnostic and prognostic marker is currently a subject of intense investigation. The further development of the various methods for detection /measurement of MP and prospective clinical trials establishing the utility of such tests will be critical prior to the routine use of measurement of MP in the diagnostic laboratory.

Key words: Microparticles, flow cytometry, disease, limitations, methods

INTRODUCTION

Cell membrane bound particles of less than a micrometer in diameter or 'microparticles' (MP) and their various functions have come to be recognized in the last few decades. [1-4] Their function as vectors of transcellular exchange of biologic information in addition to better described forms of intercellular communication such as growth factors, cytokines and chemokines, is becoming increasingly clear as is their clinical significance as biomarkers for cellular activation and /or apoptosis .[5] Circulating blood cells as well as endothelial cells release membranous fragments ~1μm in size or smaller, bearing at least some features of the parent cell. Several physiological mechanisms result in the production of MP such as activation, complement mediated lysis, shearing stress, oxidative injury and active vesiculation. MP are different from exosomes (0.03-0.1 μm), which instead originate through the exocytosis of multivesicular bodies and play a role in antigen presentation.[6]

Circulating MP levels are thought to reflect a balance in cellular homeostasis. Abnormal amplification of MP production may lead to a pathological state, for instance, excessive MP from platelets may contribute to thrombosis.[7] The following discussion will focus on the physiological (or health) and the pathophysiological (or disease) roles of MP. It will also focus on the ability of MP to function as miniature markers, their clinical utility and a discussion regarding their transition from the bench to the bedside.

MICROPARTICLES IN HEALTH

Microparticles production

The production of MP is thought to predominantly occur by vesiculation or blebbing of the cell membrane. The mechanisms governing the remodelling of the plasma membrane are complex, involving cytoskeletal changes and a shift from normal

phospholipid asymmetry. It is generally acknowledged that sustained increase in intracellular calcium is necessary to allow transmembrane redistribution of phosphatidylserine (PS) to the cell surface. Basic intracellular signalling pathways are thought to lead to cytoskeletal reorganization and formation of vesicles.[7] After stimulation, calcium is released from intracellular stores. Several candidate proteins might be involved in the process of intracellular calcium mediated cell membrane instability, including gelsolin and calpain. The latter is believed to cleave actin-capping filaments while the former cleaves long actin filaments. [7] Several enzymes including translocase, floppase and scramblase are actively involved in aminophospholipid distribution and stability on the membrane surface as depicted in Figure 1a .[8] The function of translocase is to specifically shift phospholipids from the external leaflet to the inner leaflet, whereas scramblase bidirectionally moves all phospholipids (see figure 1b).[9] As the cleaving of actin filaments occurs with the raised intracellular calcium, this also activates scramblase while inactivating translocase. As illustrated in Figure 1c, this leads to cytoskeletal instability due to disruption of the protein anchorage, as well as PS reorganization, and leads to MP formation. [8]

Microparticles in thrombus formation

The tissue factor (TF) based model for thrombosis suggests an active role for MP and P-selectin action in thrombus formation.[10] TF is expressed in the subendothelial membrane and when damage to the vessel wall occurs a significant amount of TF is released into the bloodstream in form of MP. This exposure of TF triggers the coagulation system and initiates the formation of a clot. Activated platelets bearing P-selectin are attracted to the site of injury and serve to further recruit MP to the thrombus by increased thrombin generation at the site of injury.

Other physiological roles for microparticles

Several other roles have been ascribed for MP, including enzyme and messenger functions. In contrast to the discussion in the previous section, some MP have been observed to also have anticoagulant properties (such as MP with protein C activity).[11]

MICROPARTICLES IN DISEASE

There are a number of conditions associated with elevated MP (Table 1); most are also associated with an increased risk of thrombosis. The following discussion focuses on the individual disease states in which increased levels of circulating microparticles have been reported and the possible relationship to the underlying pathology.

Trauma and Sepsis

Trauma and sepsis result in major perturbations of the coagulation system that often lead to life-threatening disorders such as disseminated intravascular coagulation and /or consumptive coagulopathy. In both events there is physical and /or chemokine induced damage to the endothelium.[12,13] Correspondingly, there are markedly raised levels of endothelial, platelet and leukocyte MP.[12,14] Whether these MP are only consequences of the vascular injury or actual mediators of ongoing pathophysiological processes and contribute causally to disease is not clearly understood.[15] Recent studies suggest a possible role for measurement of MP to predict outcomes in patients with sepsis admitted to the intensive care unit.[16] One of the possible parameters purported to be of clinical significance is the rate of clearance of microparticle-microorganism complexes by activated monocytes.[17]

Venous thromboembolism

MP have been shown to be increased in both deep venous thrombosis (DVT), as well as pulmonary embolism. A combination of D-dimer assay, p-selectin and total MP levels in blood were found to correlate significantly in patients with symptomatic DVT

documented by doppler ultrasound in a small pilot study.[18] However, the sensitivity of this combination was only 73% suggesting the need to improve the technology, or the range of markers used, to be useful in clinical and diagnostic practice.[19-21] The use MP levels in specific thrombophilic states to predict the occurrence is currently being investigation.

Hemolytic anaemias

Increased levels of MP have been observed in sickle cell disease, thalassemias and in paroxysmal nocturnal haemoglobinuria.[22,23] Increased levels of endothelial MP have been seen in all the above three hemolytic states implying endothelial activation as a major mechanism which contributes to the prothrombotic state in these conditions. [24]*Cardiovascular disease*

Endothelial activation and vascular inflammation are common pathways in the vascular complications of diabetes, hypertension, cerebrovascular disease and coronary artery syndromes[25-30]. MP arising from activation of endothelial cells may be theoretically important in this inflammatory process and may contribute to thrombosis in these patients[31]. In fact, MP have been also observed to be significantly elevated in acute coronary syndromes and in ischemic strokes.[32-38] Peripheral arterial disease has also been linked to elevated MP levels.[39] Marked elevation in endothelial and platelet MP in severe uncontrolled hypertension has been observed, although long-term follow up studies are required to validate their utility in predicting end organ damage in this clinical setting.

Myeloproliferative disorder

Elevated MP have been reported in polycythemia vera, essential thrombocytosis and myelofibrosis. Increased levels of platelet MP appears to correlate with platelet dysfunction and increased risk of thrombosis in these patients.[40,41] Given the lack of markers apart from full blood counts to predict the occurrence of venous

thromboembolism (VTE) in these conditions, measuring MP may prove to be a useful tool in follow up of patients with myeloproliferative disorders.

Thrombotic microangiopathies

Acute thrombotic thrombocytopaenic purpura (TTP) is characterized by microvascular injury and triggering of *in vivo* coagulation. Elevated levels of endothelial MP bearing von Willebrand factor and increased calpain activity has been observed in this group of patients.[42] Elevated endothelial and platelet MP have also been observed in thrombotic microangiopathy in the setting of allogenic bone marrow transplants [43].

Another distinct but pathophysiologically related entity in terms of platelet and vascular activation is heparin induced thrombocytopaenia. Elevated levels of platelet MP have been shown in this disorder and thought to relate to thrombosis in the setting of thrombocytopaenia and platelet activation.[44,45]

Pregnancy loss and pre-eclampsia

Elevated total MP have been observed in normal pregnancy [46].This isn't surprising, since the changes occurring in the haemostatic system during pregnancy shift towards a procoagulant state. The elevation of MP (both platelet and endothelial) has not been consistently reported in patients with pre-eclampsia [47]. One would theoretically expect these as placental beds are rich in phospholipid and any placental pathology would have significant procoagulant activity such as increased generation of MP.

In women with recurrent pregnancy loss, total MP has been observed to be elevated greater than 2 standard deviations from the mean of healthy parous women. This suggests that MP, being important role players in thrombosis, may play an important role in placental thrombosis and infarction leading to a wide spectrum of pregnancy complications.[48-50]

Antiphospholipid antibody syndrome

Several studies have reported elevated levels and functional capacity of MP generated in plasma of patients with antiphospholipid syndrome.[51-53] These and other reports suggest a procoagulant role for MP in the antiphopholipid syndrome as well as in other autoimmune disorders such as systemic lupus erythematosis. [54,55]

Platelet storage lesion

It has been consistently shown that more PMP are generated in platelet bags intended for transfusion as a consequence of the increased storage time.[56] This is being used as a tool to monitor platelet bags and their shelf-life.[57]One study has suggested that increased MP in blood products may be potentially pathogenic and may be associated with transfusion related reactions. [58]

Microparticles and Cancer

The MP is thought to reflect a balance between cell stimulation, proliferation and death. It is conceivable that circulating MP shed from epithelial cells and their interaction with vascular endothelium influences tumour metastasis.[59] A recent report states that elevated MP was associated with metastases in osteosarcoma patients. [60] Some MP are reported to be pro-angiogenic and are believed to act as transcellular vectors to promote or regulate angiogenesis.[61] Endothelial MP have been shown to have a metalloproteinase effect, while platelet MP seem to exert this effect by their influence on the extracellular signal related kinase pathways.[62] It has also been observed that lymphocyte derived MP can have a negative regulatory effect on angiogenesis.[63]Some reports have attempted to correlate chemotherapy responses with changes in MP levels, especially after intensive chemotherapy, such as in allogenic bone marrow transplant.[43,64,65] There appears to be an increase in MP with chemotherapy, presumably due to increased cell death, which decreases over a

period of time. Procoagulant properties of tumor cell MP have been an area of intense research. There appears to exist a positive correlation between circulating MP and cancer associated thrombosis.[66] A range of endothelial, monocyte and leukocyte MP along with tissue factor bearing MP appear to have a prothrombotic potential and have shown to be elevated in various cancers such as melanoma and prostate.[67,68]

Scott Syndrome

Theoretically, underproduction of MP should lead to a pathological state, given the significant role of microparticles in thrombus formation. Scott syndrome is a rare congenital bleeding disorder, which has been characterized for the presence of decreased microvesiculation and phosphotidyl serine exposure.[69] This is now supposed to be due to an underlying deficiency in phopholipid scramblase enzyme. This is the only well described disorder in the literature sustained by a defect or deficiency in MP production .[70]

Miscellaneous disorders

Increased MP levels have also been reported in a variety of inflammatory or chronic inflammatory conditions. Renal failure, especially patients on dialysis, have moderately increased levels of endothelial MP.[71] Diabetes mellitus is another example of a disease which affects the endothelium and shows an increased level of circulating MP.[20]In particular, increased monocyte and platelet MP are observed in diabetic patients with retinopathy as well as nephropathy.[72-74] This suggests a possible role for MP in the vascular pathology, especially neovascularization that underlies the pathophysiology of diabetes.

CLINICAL UTILITY OF MEASURING MICROPARTICLES

The role of MP in pathophysiology of various disease processes is not fully understood and is being extensively investigated. Although MP have been shown to be elevated in wide range of pathophysiologic states, they are phenotypically and functionally heterogeneous. They may prove useful as biomarkers in evaluating specific situations such as endothelial dysfunction and prothrombotic states.

The most exciting prospects are for prediction of VTE and pulmonary embolism, as well as in cardiovascular disease. The accurate diagnosis of VTE continues to be a challenge, the initial approach still relying on a combination of clinical and laboratory criteria.[75,76] The results of a pilot study showed limited sensitivity of measuring MP in predicting DVT or pulmonary embolism, but potential to be a useful marker should be combined with other diagnostic methods. The utility of MP measurement as a part of the diagnostic algorithm for DVT/PE needs to be explored further.

MP as a biomarker for management and prognosis of acute coronary syndromes is an area of great interest. Given the range of existing markers for acute coronary syndrome, especially serum markers such as troponin or creatinine kinase isoenzyme MB, it is unlikely that measuring MP will be useful in this setting. Their potential use in monitoring efficacy of specific antiplatelet agents needs is of potential interest. Circulating MP have also been evaluated as a marker of vascular endothelial damage. Pilot cross-sectional studies showed a marked elevation in endothelial and platelet MP in severe uncontrolled hypertension, although long-term follow up studies are required to validate their utility in predicting end organ damage in this clinical setting.

MP of cancer cell origin as an early marker for detection/ prognosis of malignancies is also being explored. This area is still in embryo, with some reports suggesting that MP might influence tumour angiogenesis and possible distant metastasis. MP have been reported to be a peripheral blood marker for monitoring chemotherapy responses or specific situations such as allogenic bone marrow transplants. Cancer related thrombosis is another area of intense investigation, where MP could be developed as a potential tool.

As the role of microparticles in pathophysiology of disease is better understood, it may be possible to develop treatment strategies against specific targets carried by MP such as p-selectin.[77] The current research on MP thus involves many different aspects including the possibility of the exciting opportunity for targeted therapy.

TECHNICAL CHALLENGES FOR MEASUREMENT OF MP IN THE CLINICAL LABORATORY

In order to be clinically useful in all the different scenarios previously described, the preanalytical and analytical components of MP measurement need to be accurately standardized. An international group is already working towards the achievement of this task (the vascular biology subcommittee of the International Society of Thrombosis and hemostasis).[78] MP assessment by standardized techniques, validated by prospective studies and clinical trials, will provide the basis for their use in routine clinical practice. In the present time, however, their use as a research tool, and as a marker for activation of circulating/endothelial cells, will provide useful information regarding global vascular responses and signaling mechanisms within the vascular system.

There are several technical limitations for each of the existing strategies for detecting microparticles. A detailed discussion of the current methodology for detection of MP, can be found in an earlier review by the authors, as well as in other excellent articles.[79-81] Only certain limitations in the major methods in current usage, which continue to be challenges for a transition from a bench to the bedside application of MP measurement will be discussed in the following section.

Flow cytometric analysis of microparticles

The detection of submicron sized particles using any method is a technical challenge. The ability to accurately quantify such elements is a further stretch of present technological boundaries. In most multiparameter flow cytometers, the small-angle (forward scatter measurements) indicates the size of a particle. However, a number of other factors can influence this measurement, such as the refractive index, internal structure, suspending medium, presence of other cellular material, wavelength and range of angles over which the scatter light is recorded.[82] The current generation of flow cytometers will be able to efficiently detect signals in the MP size range using a combination of forward and side scatter. Standardizing forward scatter, inclusion of all particles in the appropriate size range remains a challenge for accurate quantitative assessments of MP. One possible approach is the use of standardized beads of a known size to appropriately set gates for detection of MP.[83,84]

The discrimination between MP and background electronic ‘‘noise’’ of the flow cytometer is another technical problem. Distinction on the size basis alone is not useful, as the two often appear as events in the same region with most flow cytometers. Thus, forward and side scatter alone can be used to distinguish MP from larger cells or particles, but are not enough accurate to correctly identify and quantify MP. This would require a third parameter, which can be provided by the use of an antibody to detect a particular antigen (such as CD41 on platelet MP), the use of a

generic marker (such as annexin V to identify the expression of phosphatidylserine on the surface of the MP cell membrane), or a more global biological marker (such as maleimide or annexin V).[85] When conjugated to a fluorophore, the resulting fluorescence can be detected by the flow cytometer and allow the differentiation of biological MP material from background electronic noise.

Enzyme linked immunoassays (ELISA)

The Enzyme Linked Immunosorbent Assay (ELISA)-based techniques are not limited by the size issue, as the detection of larger particles has been prevented by initial centrifugation and filtration techniques. However it is possible that ELISA detects soluble as well as MP based proteins. Moreover, since ELISA cannot quantify more than one protein at a time, the presence of multiple markers on MP of interest cannot be elucidated and this continues to be a major challenge.

Procoagulant assays

A few procoagulant assays have been described for detection of MP. One commercially available assay is based on a modified Xa assay, measuring the procoagulant activity of the phospholipid content of the plasma.[86] One drawback of this technique is it's the inability to measure free MP in contrast to membrane bound and free circulating MP that may be present in the plasma.

Combination assay

A combination of two or more principles of detection, such as a commercially available assay which combines an ELISA with a prothrombinase assay to give a measure of annexin V expressing procoagulant MP is availabel for detection and quantiation of procioagulant MP which express phosphotidylserine. A major drawback of this technique is the inability of capture MP which do not express annexin V, which is the most widely accepted generic marker for MP.[79]

Measurement of enzyme activity relevant to MP production

Several groups have attempted to measure calpain activity as a surrogate marker of MP production. As previously discussed, calpain is one of the proteins which leads to increased intracellular calcium, and further activation of enzymes that control membrane assymetry. Increased calpain activity has been noted in clinical syndromes such as TTP, where activated platelets release excess platelet MP.[42]

CONCLUSION

The association of various physiological and pathological processes with increased levels of MP has exemplified MP as transcellular messengers. After long being considered 'cell dust', MP are now considered as a surrogate markers of *in vitro* cell stimulation and transcellular vectors of information. MP may also have important roles in thrombosis, cardiovascular pathology, neoplasia and angiogenesis. Measuring MP has emerged as an important research tool in vascular biology. However, the ability of such a measurement to serve as clinically useful is subject of debate. The performance of ‘diagnostic test research’, in contrast to the quality of interventional clinical trials, continues to lack rigor in study design, process, analysis and reporting, thus hampering the traditional translation from “the bench to the bedside”.[87,88] There are three main hurdles for MP to reach the bedside as a laboratory tool. First, a clear diagnostic or prognostic role for such a measurement, which can be only be achieved by including the measurement of MP in prospective clinical trials for various disorders. Then, standardized techniques and reporting processes will be critical to allow interpretation of these measurements across studies. Finally, the ability of a chosen method to demonstrate an extra benefit to the existing range of testing available for the particular clinical condition will justify the health economics of employing this test. The continued development of the various modalities for detection / measurement of MP and rigorous prospective clinical trials

establishing the use of such tests will be critical prior to the routine use of MP measurement in the clinical laboratory.

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Table 1. Conditions associated with elevated circulating MPs.

System/ Condition	Associated pathology
Cardiovascular disease	Hypertension Myocardial infarct/angina Stroke Diabetes Thromboembolism
Myeloproliferative Disorder	Polycythemia vera Essential Thrombocytosis Myelofibrosis
Thrombotic Microangiopathies	Thrombotic thrombocytopaenic purpura Heparin induced thrombocytopaenia Pre-eclampsia of pregnancy
Antiphospholipid Syndrome	Antiphopholipid antibody syndrome Systemic lupus erythematosis
Cancer	Metastatic solid tumours Chemotherapy induced Neoangiogenesis

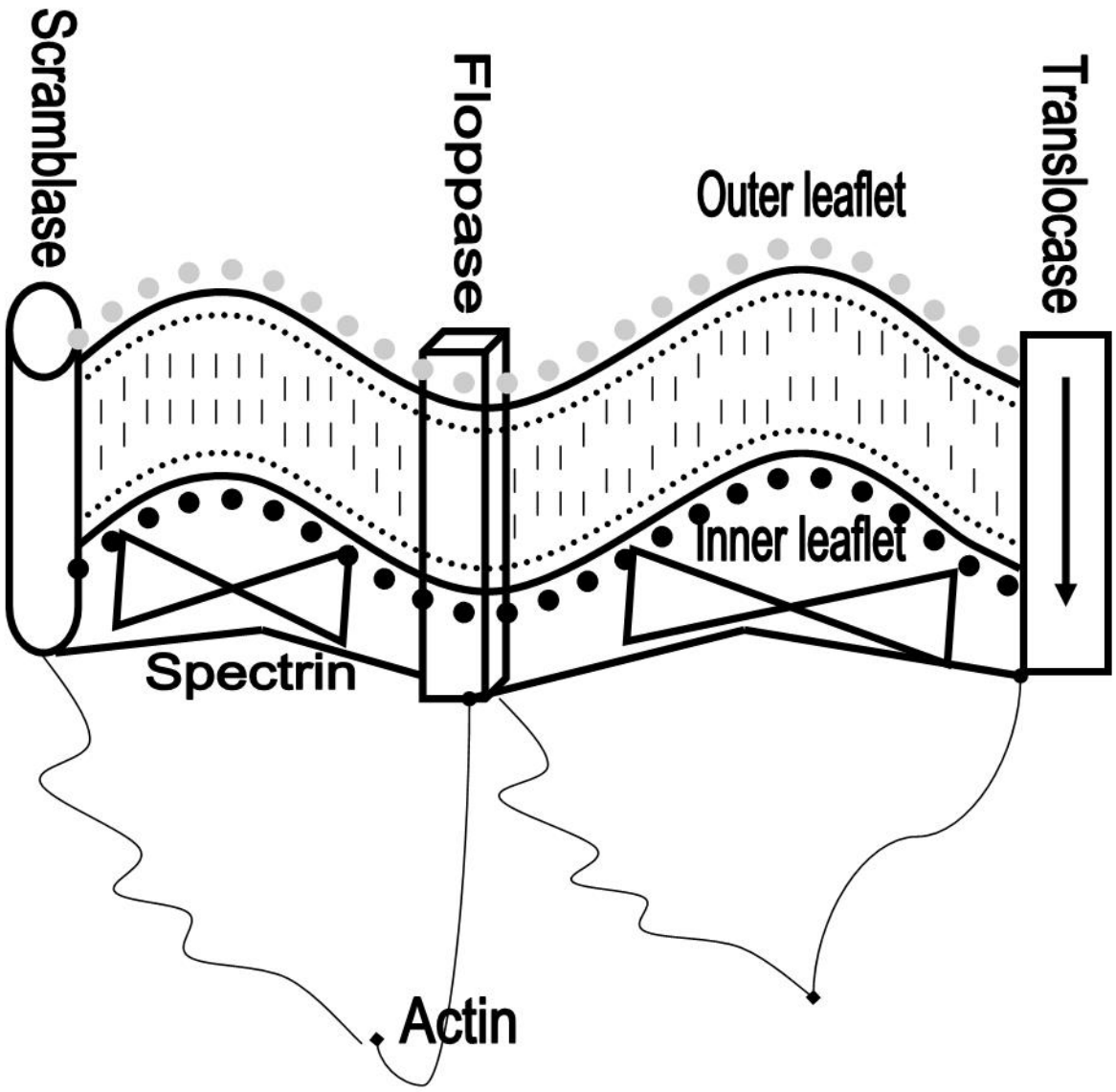
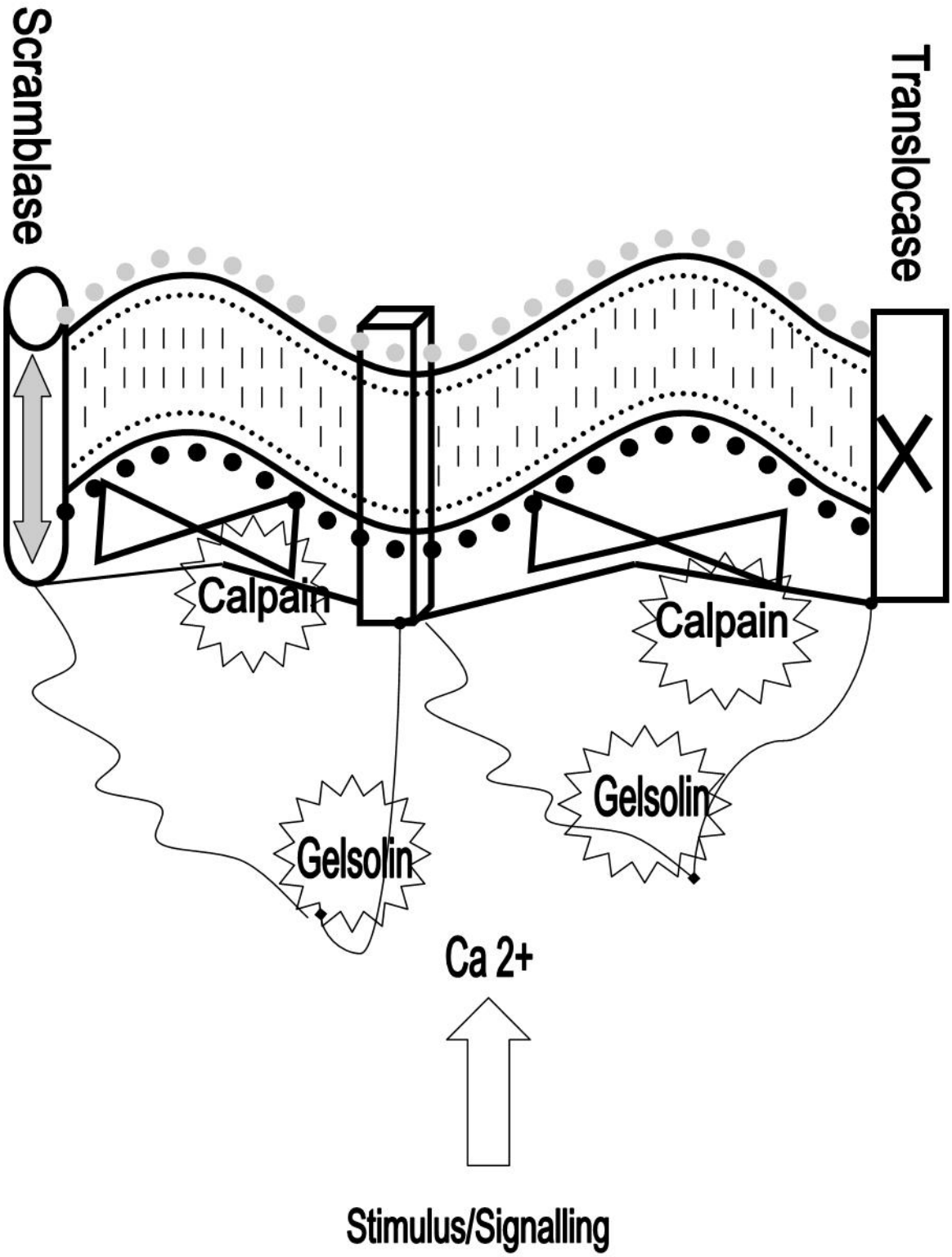
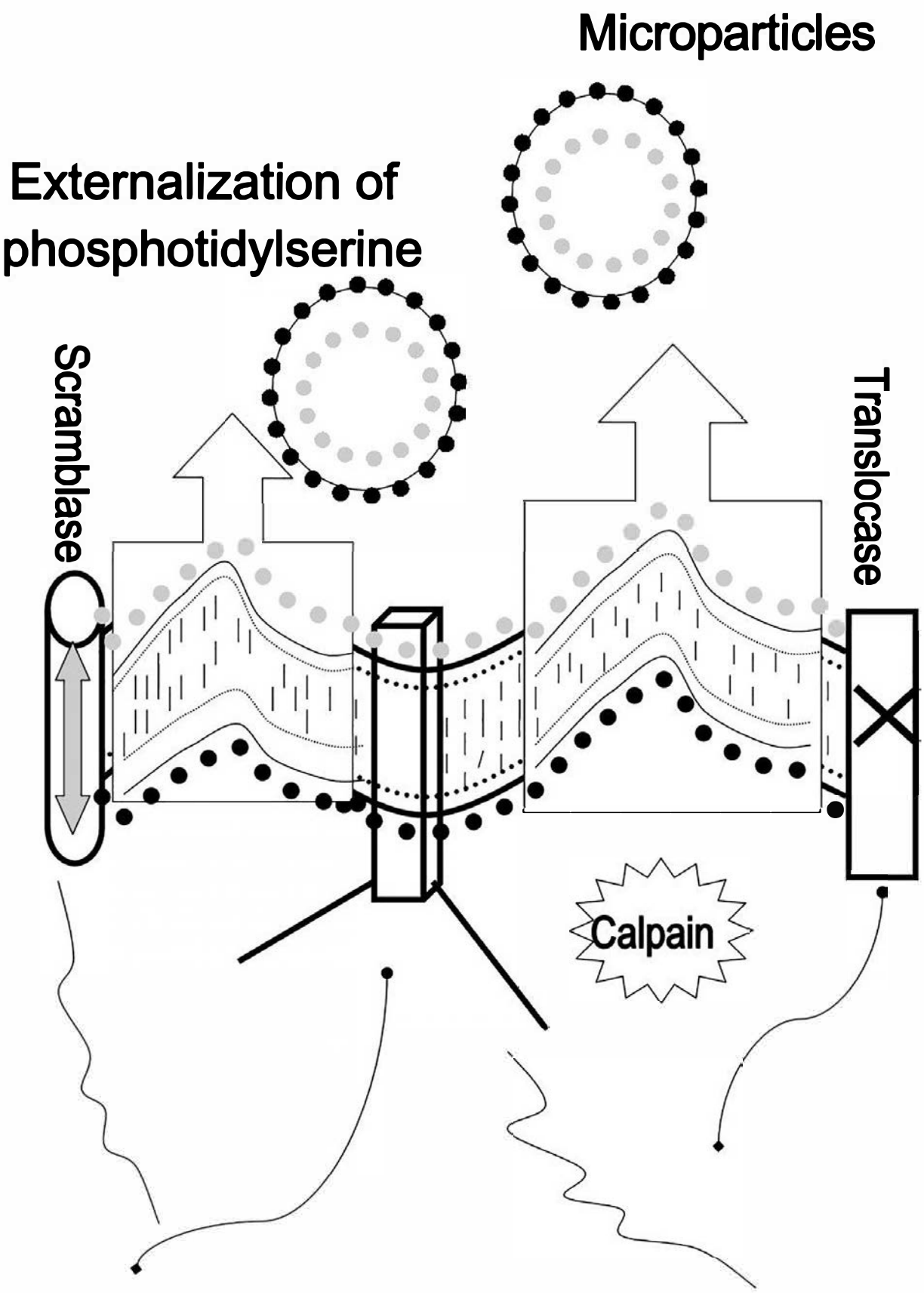

Scramblase
Floppase
Translocase
Outer leaflet
Inner leaflet
Spectrin
Actin

Figure 1. Normal membrane asymmetry is maintained by the activity of translocase (1a). This can be disrupted by a variety of stimuli which increase intracellular calcium which inturn activates gelsolin, calpain and scramblase while inactivating translocase. Gelsolin and Calpain cleave actin filaments while scramblase activity promotes membrane asymmetry (1b). This leads to externalization of phosphotidylserine and formation of microparticles (1c).


Scramblase
Translocase
Calpain
Calpain
Gelsolin
Gelsolin
Ca 2+
Stimulus/Signalling


Microparticles
Externalization of phosphotidylserine
Scramblase
Translocase
Calpain

A detailed discussion of the methods used to measure MV/EV follows this introductory chapter.

CHAPTER 3

TECHNIQUES TO DETECT AND MEASURE MICROVESICLES/EXTRACELLULAR VESICLES

3.1 Detection of MV/EV: an overview

In recent years, measuring MV has gained attention both as an investigative and potential prognostic tool. Different methods for MV assessment that have been developed include microscopy, flow cytometry, solid phase capture and enzyme linked immunoassay (ELISA).

Although a number of publications linking MV to thrombosis have been published recently, global standardization of techniques for MV evaluation is still being actively pursued.[41] The 'normal' MV count range in human plasma of healthy subjects is not clearly established and is debatable, as biological variables, both known and unknown are a source of variation. Moreover, standardising analytical (including pre- and post-test) variables remains a challenging area.[42] The appropriate selection of patient population, anticoagulant and centrifugation are some of the key pre-analytical variables.[43] Analytical variables that impact measurement include the method employed (e.g. ELISA versus flow cytometry). Post analytical variables for flow cytometry are the repertoire of antibodies used, the fluorescent label which may affect spectral overlap, data capture and analysis. The other post analytical variables which affect results are use of commercial beads and the formula for measurement counts.[44]

3.2 Prenalytical variables

3.2.1 Sample collection

In one multisite international study, the use of a well-documented sample collection and processing protocol, which includes medication and smoking history, produced comparable results amongst laboratories.[45] There is relative agreement that citrated plasma is the sample of preference for MV work. Citrated plasma has been generally the method of choice in experiments in the context of haemostasis and coagulation.[45] Plasma is also the preferred sample type for RNA work as it is the physiological medium for MV in circulation. Although serum samples may have potential uses, the clotting that occurs ex vivo has significant impacts on both quantity and function of MV in the sample interrogated.[46]

Samples collected in EDTA invariably result in platelet activation or as seen in one study suppression of MV levels. Thus, EDTA is not suitable for analysis of MV, and in particular of platelet MV.[47] In one study, however, EDTA samples for platelet MV were analysed and reported by ELISA but these have not been compared with another method or alternative anticoagulant.[47,48] Given that EDTA is cell toxic and platelet activating, it is not a suitable medium for measuring platelet MV.

The two pre-analytical issues critical to evaluation of MV by flow cytometry or ELISA are the effect of freezing and centrifugation speeds.[49] Although freezing has been reported not to affect MV counts in several studies there is has not been an extensive evaluation of this step at varying concentrations of MV. In one analysis of endothelial MV, fresh plasma samples were found superior to frozen samples.[12] The ISTH MP/MV standardization subcommittee suggests that freezing samples may not significantly affect MV counts but these recommendations need to be interpreted cautiously.[45,50-52] The updated MISEV guideline

acknowledges the lack of consensus on how to approach sample processing however point out the choice of pre-analytical approach is to be guided by the downstream application. [10]

3.2.2 Isolation of MV

MV characteristics, both antigenic and functional, need to be assessed without influence of similar sized biological vesicles such as platelets. Isolating MV is also critical for assessing their cargo such as miRNA or protein without the influence of plasma proteins or RNA. Different isolation techniques may also yield varied MV fractions of interest for further investigation.

Differential centrifugation is the mainstay of MV isolation described in the literature. Centrifugation speeds of 1500g for 30 mins results in platelet free plasma. Higher speeds between 10000 to 21000g can be achieved by bench top centrifuges and can efficiently isolate MV and larger vesicles.[53] Ultracentrifugation at 100,000g can be used to isolate exosome fractions. The reported ranges of MV levels in circulation have varied depending on the centrifugation speed and method employed. A wide variety of centrifugation speeds have been employed in an attempt to concentrate or pellet MV. Additional centrifugation at various speeds has been tried in order to simultaneously remove platelets (platelet poor plasma, PPP) and preserve the MV.[46] In cases where 1500g was the only spin employed the platelet contamination influencing the range of MV is clearly evident.[54] In fact, accurate quantification of MV by flow cytometry in these samples could be technically difficult in view of the some overlap in size between MV and platelets.

Novel approaches to MV and exosome isolation include using a column based technique rather than centrifugation. Some of the other methods have employed immuno-affinity capture, microfluidic separation, HPLC, membrane filtration and polymer based precipitation.[46] However, centrifugation remains a popular and most widely available technique for MV isolation. Most flow cytometry protocols have been performed on samples centrifuged at 1500g for 30 mins followed by a shorter spin using between 12,000g to 20,000g to effectively remove any remaining platelets.[43,54,55]

3.3 Microscopy

Particles less than 1μm are difficult to visualize using routine light microscopy. However, specialized techniques such as confocal laser microscopy and electron microscopy are able to better characterize MV. Confocal microscopy has demonstrated MV which are released upon activation of coated platelets.[56] Electron microscopy has demonstrated the presence of MV with internal morphological features similar to the parent cell but with complex internal membrane architecture.[57] In contrast to circulating lipoprotein particles, electron microscopy of MV has shown them to be heterogeneous in size and shape with most of them being true vesicles. [58]

3.4 Flow Cytometry

Flow cytometry has emerged as an important technique for detection and measurement of MV. Flow cytometry is a popular approach due to rapid turn-around time, ability to detect two or more antigens simultaneously and quantitation of MV using a known concentration of counting beads.[59] However, it does have several drawbacks. The detection of particles less

than 500nm is more challenging by flow cytometry as the detection is limited by particle size in the same order of magnitude wavelength of the laser (~488 nm). Different machines have different sensitivities and different gating strategies have to be employed to detect MV in an optimal fashion.[3,60] It requires significant expertise in pre-analytical preparation and interpretation can be subjective.[61] The detection of the MV depends on the refractive index, absorption coefficient, and particle shape properties apart from its light scattering properties. High resolution flow cytometers which are capable of detecting and visualising particles less than 200nm in size have also been described.[62] The higher resolution is achieved by detecting shifts in fluorescence of background noise and label bound particles in a more efficient manner. This nanoFACS approach also uses fluorescent labelling to define lipid, protein and nucleic acid binding EVs.[63] Although promising, such instruments or techniques are not routinely available in most laboratories. It is also important to note that, given their lipid content, EVs of biological origin have a much lower scatter than polystyrene beads. The use of polystyrene beads to determine EV gating parameters therefore carries a significant risk for uncertainty in measurement.[14]

A variety of flow cytometry markers have been used for detection of MV. These may be divided into primarily those that detect activation status and those that define the cell of origin.The common preanalytical steps and antibodies used in flow cytometry are described in table 3(i). Multicolour flow cytometry is a popular approach to define the different sets of MV in circulation. [24] Two international societies have taken up the task of standardization of the various techniques, particularly flow cytometry. The ISTH has conducted two workshops, with a third which was recently published in order to address the issues of standardization for MV measurement by flow cytometry.[64,65] The international society for extracellular vesicles (ISEV) has also published an evaluation of the various methods for detection of MV, including a collaboration with ISAC (International Society for Advancement of Cytometry) in best practices referred to as MISEV (Minimal Experimental Requirements for

Extracellular Vesicles).[15,66,67] Most recently, the ISTH, ISAC, and ISEV consortium have been working to standardize protocols and putting forth recommendations for the use of flow cytometry in EV detection (Methodological Guidelines to Study Extracellular Vesicles).[15,68]

Table 3(i). Summary of various methods for isolation and detection of MV. Adapted from reference (Enjeti et al, appendeix paper 1)[1]

Authors	Technique	Sample collecti-on tube	Centrifu-gation	Isolation of MV	Generic MV detection	Antigens to detect MV cell of origin and/or activation state		
						Platelet	Endothel-ial	Leukoc-yte
Biro *et a[41]l*	Flow cytometry	Citrate	1550 × *g*, 20 min	18 000 × *g*, 30 min	Annexin V	CD62P, CD61, CD63	CD31, CD62E or CD144	CD4, CD8
Dignat-. George *et al*[69]	Flow cytometry	Citrate	1500 × *g*, 15 min 13 000 × *g*, 2 min	None	Annexin V	CD41	CD51, CD144 or CD146	CD45
Freyssinet *et al.* [70]	Solid phase capture	Citrate	1500 × *g*, 15 min 13 000 × *g*, 2 min	None	Annexin V, tissue factor	CD62P or GPIba	CD31 or CD62E	CD45
Jimenez *et al.*[41]	Flow cytometry	Citrate	200 × *g*, 10 min 1500 × *g*, 7 min	None	None	CD41 or CD42b & CD31	CD31+/ CD 42– or CD62E	CD45
Nomura *et al*[71]	ELISA	EDTA	1500 × *g*, 20 min	None	None	GP IX (capture) CD62P, CD40L	None	None
Enjeti *et al*[72]	Flow cytometry	Citrate	1500 x g,30min 13000 x g, 2min	None	Maleimide	CD41	CD62e	CD45
Dale *et al*[48]	Flow cytometry	Citrate	Platelet rich plasma	Activation of coated platelets	maleimide	GPIIb/IIIa	None	None
Shet *et al*[57]	Flow cytometry	Citrate	13 000 × *g*, 10 min	100 000 × *g*, 60 min	Annexin V	CD41a	CD144	

3.4.1 Characterization of surface antigens on MV

Commonly used antibodies for detection of MV are listed in table 3(ii). Most of these can be used in either flow cytometry or ELISA platforms.

Table 3(ii). List of antibodies against cell specific antigens for detection of MV from blood. Adapted and updated from reference (Enjeti et al, appendix paper 1)[1]

Cell Specificity	**marker**
Generic	Annexin V Lactadherin Maleimide (binds to PS, flow cytometry only) Tissue Factor
Platelets	CD62p(P-Selectin) CD42
Endothelium	CD31 CD105 CD62e (E-Selectin)
Monocytes	CD14
Lymphocytes	CD3/CD4 (T lymphocytes) CD20 (B lymphocytes)

3.4.2 Quantification of MV by flow cytometry

MV may be quantified by the following methods in flow cytometry:

1. As a proportion of the platelets in the sample, this is particularly suitable for platelet derived MV or MV released upon platelet activation.[57]

2. As an absolute number calculated from a known quantity of commercial beads added to the sample.[60]

3. Calculation by aspirating a known amount of sample for analysis.

4. Expressed in terms of fluorescence compared to standard flourospheres.

When using ELISA techniques, MV may be quantitated with reference to a standard curve created by using liposomes containing a known quantity of the antigen of interest. Some of the analytical variables for platelet MV including comparison of specific antibodies and linearity have been evaluated.[45,46,50-52,73] Antibodies may vary in affinity based on the clone used and type of fluorophore linked to the antibody. Linearity and optimal dilution of antibodies should be determined prior to commencing experiments.[1]

3.4.3 Gating strategies

There could be several approaches to gating MV adequately on flow cytometers. Most of these use beads of fixed sizes to create a 'size gate' within which the MV events that fulfil the size criteria will fall. 'Megamix beads' is one such approach to standardizing the gating of MV by flow cytometry. It uses a mix of a 0.9um and 0.3um sized beads to capture all events within the gate set by the beads.[21] One of the problems of using beads is the lack of linearity in the relationship between the size of beads and forward scatter at that particle size (i.e. <1 μm). It is therefore recommended that to overcome this problem, one uses side scatter discrimination, particularly whilst using instruments such as BD FACS Canto.[45] Isotype controls in flow cytometry for MV is also a subject of discussion due to non-specific binding of antibody to MV.[46] The issue of variability between observers, instruments and laboratories in flow cytometric analysis of MV was addressed in the second and third standardization

workshops by ISTH.[45,65] The international society for extracellular vesicles has also published on various aspects of sample collection, isolation and analysis methods in extracellular vesicle research.[10,46]

The MV were enumerated by flow cytometry using the following formula

MV /µl = (MV gate events/Bead gate events) x (total bead count/volume of the sample in µl)

3.5 Enzyme linked immunoassays/capture assays

In comparison to flow cytometry, antigen capture assays using the enzyme linked immune-absorbent technique (ELISA) has been less commonly used. The capture of MV into immobilized annexin V or cell specific antibodies have been the major approaches. Solid phase assays have the advantage of picking up MV irrespective of size.[74] However interference of soluble antigens, variable quality of antibodies employed for antigen capture and non-exclusion of exosomes are some of the disadvantages. Table 3(iii) compares Flow cytometry and solid phase assays.

In one of the more commonly used approaches, annexin V is insolubilized onto streptavidin-coated ELISA plates is used for capturing phosphotidylserine expressing MV. Platelet-free plasma is then added, incubated and washed.[75] Background values obtained with irrelevant IgG are subtracted from those measured with specific antibodies. Tissue factor based ELISA assays are also now available and are also widely in use. Antigen specific capture assays will measure only that fraction of MV which are antigen expressing .[76]

The use of a multiplex bead array to analyse the profiling of cytokines and proteins associated with MV/EV has also been recently described. This study reported that cytokine

profile of EVs can vary with the activation trigger for a given biological system rather than any specicific association with a type of EV. [26]

Table 3(iii). Comparison of Flow cytometry and ELISA techniques to detect and measure MV. Adapated and updated from reference (Enjeti et al, appendix paper 1)[1] [48,55]

Variable	**Solid Phase capture assays**	**Flow Cytometry based assays**
Type of antigen	Measures soluble and membrane bound antigens	Measures only membrane bound antigens
Size	No lower limit for size detection	Size detection limited by wavelength of light used probably around 200-300nm
Number of antigens	Can detect only one antigen expression at a time	Multiple antigens on the same MV can be detected by multicolour flow cytometry
Functional assessment	Can combine antigen detection with a functional assay	Only surface antigen expression detected
Sensitivity	Sufficiently sensitive to pick up weak antigenic expression	Limited ability to pick weakly expressed antigens such as endothelial antigens
Cost and ability to automate	Although more labour intensive if doing an in-house assay Easier to automate once established or if using a kit	More difficult to automate

3.6 Functional assays **(***clot based assays)*

Microvesicles produced as a result of cell activation usually express phosphotidylserine on the cell surface. A novel factor X-activated clotting time (XaCT) has been recently described, the results of which seem to correlate with the annexin V binding MV by flow cytometry.[77] Other clot-based assays such as a dilute APTT (activated partial thromboplastin time) could potentially be used for determining the procoagulant function of membrane bound phospholipid in plasma samples. Baseline coagulation tests such as prothrombin time(PT), d-dimer and fibrinogen assays which measure various aspects of the coagulation pathway are not sufficiently sensitive to detect MV levels. The contribution of the soluble fraction of phospholipid in these tests is not entirely clear.[48] The correlation of clot based tests with various subsets of MV is not clearly established. It is likely that a particular subset such as platelet MV preferentially influences the clotting assay.[78]

Thrombin generation is a technique that measures the generation of thrombin, which is the end product of coagulation, using an semi-automated fluorometric method.[79] It measures molar concentration of thrombin in the clotting plasma by subsampling the plasma sample and estimating the thrombin generated.[79] The thrombin generation curve has a short lag phase followed by a propagation phase and subsequent termination due to inhibition by antithrombin. A software program displays this curve for thrombin activity monitored continuously and the area under the curve, which is the key measurement and is referred to as endogenous thrombin potential. Each test on this semi-automated technique, known as calibrated automated thrombography (CAT), depends on comparing two curves which relies on use of a low affinity fluorogenic substrate. Tissue factor and synthetic phospholipids are added to the test well and a known amount of a thrombin calibrator with defined substrate converting activity is added to a separate calibration well in a microtitre plate. A mix of calcium chloride and fluorogenic substrate is then added to the wells and thrombin

generation is monitored in real time. The slope of the calibration well tracing provides a factor that can used to determine thrombin concentration from fluorescence units.[79] The software then uses mathematical algorithms to determine thrombin generation in the test well [see figure 3(a)]. The test reagent has been modified by the manufacturer to suit measuring thrombin generation from MV and this technique has been evaluated to detect procoagulant MVs.[80]

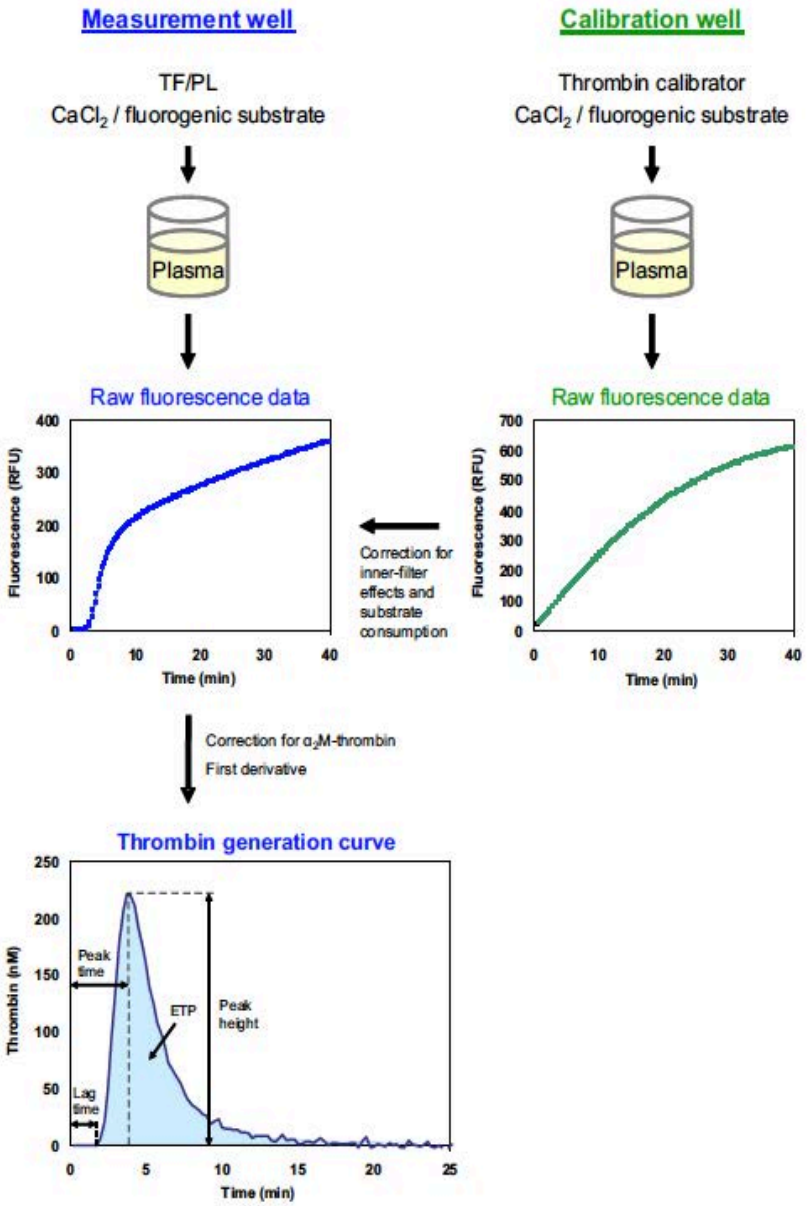


Figure 3(a). Typical curves for thrombin generation in measurement and calibration wells. Reproduced from reference.[79]

3.7 Combination of functional and capture assays

Enzyme linked immune-absorbent (ELISA) in combination with functional assays has been used to detect and measure MV from different cellular origins. This essentially employs capture of MV which are phosphatidylserine expressing as the first step. In a subsequent reaction, the phosphatidylserine content of the bound MV is measured through its ability to promote the activation of prothrombin to thrombin. The blood clotting factor concentrations are adjusted to ensure that this phospholipid is the rate-limiting parameter of the reaction. Immobilized MV are incubated with factor Xa, factor Va, prothrombin and $CaCl_2$ in buffer.[81] Chromogenic substrate for thrombin is added and linear absorbance changes are recorded at 405 nm using a micro-titre plate reader equipped with kinetics software. Results are expressed as nano-molar phosphatidylserine equivalent by reference to a standard curve constructed by using liposomes of known concentration, containing phosphatidylserine and phosphatidylcholine in a 1:2 ratio.[81] Commercially available kits have simplified previously employed in house ELISA based assays. The major advantage is the functional clotting potential assessment. However, the selection of MV by annexin V binding and quantitation by a prothrombinase assay measures selectively only a subset of MV. The overlap of size range between 'small' platelets and some MV by flow cytometry and inability of ELISA to separate soluble and membrane bound antigens is a source of difficulty in measurement. [48]

Appendix PAPER 2 (Review):

Enjeti AK, Lincz LF, Seldon M. Detection and measurement of microparticles: an evolving research tool for vascular biology. *Semin Thromb Hemost*. 2007;33(8):771-779.

Key learning points

Review of various methods used for MP enumeration was undertaken(carried out prior to starting the PhD).

Note: Due to the accepted manuscript being included in final thesis for Open Access purposes, the page numbers in the thesis are not sequential.

Title: Detection and Measurement of Microparticles: An Evolving Research Tool for Vascular Biology

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ABSTRACT

Microparticles are small membrane bound vesicles that are generated from cells of different origin. It now appears that all circulating blood cells as well as endothelial cells release membranous fragments ~1um in size or smaller bearing at least some characteristics of the parent cell. Elevated levels of microparticles have been described in cardiovascular states, thrombotic conditions and cancer. Various methods of detection for microparticles include flow cytometry, enzyme linked immuno-assays and functional assays. Flow cytometry has several advantages including its ability to quantitate and identify microparticles of different cellular origin. However, the standardization of pre-analytical and analytical variables for enumeration of microparticles remains a significant challenge. Newer approaches are being investigated and an international collaboration is working on standardization of detection as well as quantitation of microparticles by flow cytometery. Although it has evolved as an important vascular biology research tool, MP detection needs further evaluation and refinement before it becomes truly useful as a clinical tool.

Key words: Microparticles, detection, measurement, research tool, vascular biology

INTRODUCTION

Microparticles (MP) are small membrane bound particles which are shed from a cell surface. These are released from activated or dead (apoptotic) cells.[1] After long being considered cell 'dust', MP have been shown to reflect *in vitro* cell stimulation and have been identified *in vivo* under a variety of conditions.[2] It now appears that MP have considerable pathophysiologic potential, with increasing evidence that they may interact with near or remote cells. MP may be regarded as vectors of transcellular exchange of biologic information.[2] It has been shown that all circulating blood cells as well as endothelial cells release membranous fragments ~1um in size or smaller bearing at least some characteristics of the parent cell. They differ from exosomes (0.03-0.1µm) which originate through the exocytosis of endocytic multivesicular bodies and play a role in antigen presentation.[2,3] Production of MP may be caused by activation, complement mediated lysis, shearing stress, oxidative injury and active vesiculation.[2] The relative sizes of platelets, microparticles and exosomes are demonstrated in figure 1.

Although the first reports regarding the existence of MP were made several years ago,[4] there is still considerable debate about how best to detect and measure them. This chapter provides a review of the current status of understanding concerning technical aspects of MP detection and measurement and their possible role as a clinical, diagnostic, and/or research tool.

DEFINING A MICROPARTICLE

The International Society on Thrombosis and Haemostasis (ISTH) subcommittee in vascular biology has developed the following definition for MP (Minutes of the SCC vascular biology meeting, Sydney, August 2005):

1. Size 0.1- 1 um.

2. Lack a nucleus or synthetic capacity
3. Contain a membrane cytoskeleton
4. May arise from a variety of cells and contain a variable amount of surface phosphatidylserine.

This definition is somewhat arbitrary and the size criteria probably reflects the limit of detection for most methods currently used to identify membrane bound particles. In reality, MP probably represent a spectrum of different sized particles ranging from very small (less than 0.1 um) to fragments as large as platelets. In fact, membrane bound particles have long been noted by anatomical pathologists in their specimens from lymph nodes or tumor tissue. In fine needle aspirates from lymph nodes, cytoplasmic fragments derived from lymphocytes known as lymphoglandular bodies have been observed.[5,6] Thanatosomes, which are eosinophilic hyaline globules noted on tissue sections in solid tumors, are probably another example of particles shed from apoptotic cells.[7] It is possible that both lymphoglandular bodies and thanatosomes are produced by similar pathophysiological mechanisms to those resulting in MP (such as activation or apoptosis) but the particles produced are substantially larger, probably several microns in size, in comparison to conventional MP. For the purpose of this chapter, the ISTH definition will be adopted as it best describes the particles of interest within the human circulatory system.

PRODUCTION OF MICROPARTICLES

The production of MP is thought to predominantly occur by vesiculation or blebbing of the cell membrane. The mechanisms governing the remodeling of the plasma membrane are not known. It is generally agreed that a sustained increase in intracellular calcium is necessary to allow transmembrane redistribution of phosphatidylserine. Basic

intracellular signaling pathways are thought to lead to cytoskeletal reorganization and formation of vesicles. The vesiculation appears to be an energy (ATP) dependent process[8] and may be viewed as an externalization of physiologically important molecules to the cell or membrane surface (Figure 2). Such molecules may include phosphatidyl serine, GPIIb-IIIa and adhesion proteins. Production of MP is thought to reflect a balance between cell stimulation, proliferation and death. Abnormal amplification of MP production may lead to a pathological state. For example, excessive MP from platelets may contribute to thrombosis.[9] Based on their potential function and pathophysiologic effect, MP are thought to be either 'bad' (deleterious effect) or 'good'(beneficial effect).[1]

CLASSIFICATION OF MICROPARTICLES

MP can be classified in different ways based on their inherent properties. An outline of the various categories of MP is shown in Table 1. The multifaceted MP has been aptly described as a 'miniature envoy with many faces'.[2]

WHY MEASURE MICROPARTICLES?

As outlined above MP are thought to participate in a variety of pathophysiological processes. However, the evidence for a clear association and role in specific disease is limited. The most widely studied aspect of MP are probably their procoagulant effects and even then it is interesting to note that that not all MP are procoagulant.[10,11] Levels of MP are higher in patients with venous thrombosis as well as arterial thrombosis when compared to healthy subjects.[1] In fact, a recent review has emphasized MP as key players in thrombosis by including them as a part of coagulation triad.[12] Thus, quantifying MP may be important in assessing the thrombogenic profile of an individual. However, the association of MP with prothrombotic processes such as deep vein thrombosis, strokes and cardiovascular conditions[13,14] is mainly on the basis of

circumstantial evidence. The use of MP quantitation as a clinical tool is still a matter of debate, with several ongoing clinical studies addressing this issue.

In contrast to the lack of adequate information in specific clinical states, the role of MP in research is more clearly emerging. The possible applications for detection and measurement of MP for *in vitro* or *in vivo* models include:

1. Detection of activated endothelial and or platelets cells.[15-18]
2. *In vitro* thrombus formation models.[19-23]
3. Measuring activation of endothelium and subendothelium in models of atherosclerosis and arterial thrombosis.[24-27]
4. Detection of leukocyte activation in disease models (including thrombosis) involving leukocyte activation.[28-32]
5. Measurement of 'storage lesions' such as in transfusion products stored over a period of time or various transfusion delivery devices and their impact on blood products by marking MP of interest.[33-36]
6. Surrogate measure for red cell hemolysis such as in inherited hemolytic anemia (e.g. sickle cell anemia).[37]
7. Detection of activation or injury to cells in a culture system.[18,30,38,39]
8. Evaluation of cancer related thrombosis or cancer micrometastasis or neo-angiogenesis models.[40,41]
9. Evaluation of autoimmune disease models such as heparin induced thrombocytopaenia,[42,43] autoimmune thrombocytopaenias[44] and antiphospholipid antibody syndrome.[16,42,45]
10. Evaluation of micro- or nano-sized drug delivery systems, some of which are relevant to treating cardiovascular disease states or use MP derived from the circulatory system.[46-51]

The extensive use of MP measurement in the vascular biology research laboratory has seen it emerge as an important tool to assess activation or apoptosis of cells in the circulatory system. Being a biomarker for cellular activity, its potential use includes areas such as cancer cell activation and death. These roles are still being actively explored and will depend on the availability of a robust antibody for marking these cells by standard techniques used for measuring MP.

TECHNIQUES TO DETECT AND MEASURE MICROPARTICLES

Detection of Microparticles - overview

In recent years, measuring MP has increasingly received attention both as a diagnostic aid and investigative tool. Different methods have been employed including microscopy, flow cytometry, solid phase capture and enzyme linked immunoabsorbent assay (ELISA). Table 2 highlights the most widely used methods.

Microscopy

Particles less than 1µm are difficult to visualize using routine light microscopy. However, specialized techniques such as confocal laser microscopy and electron microscopy may help to better characterize these particles. Confocal microscopy has demonstrated the presence of MP in platelet rich plasma, which are released upon activation of coated platelets.[57] Electron microscopy has demonstrated the presence of MP with internal morphological features similar to the parent cell but with complex internal membrane architecture.[8] In contrast to circulating lipoprotein particles, electron microscopy of MP has shown them to be heterogeneous in size and shape with most being true vesicles.[58]

Flow cytometry

Flow cytometry has emerged as an important technique for detection and measurement of MP, primarily for the following reasons:[8,59]

1. Rapid turn around time
2. The expression of two or more antigens on the MP may be simultaneously demonstrated.
3. Easy method for quantification using commercial beads.

However this technique also has the following drawbacks:

1. The detection of particles less than 0.5μm is difficult by flow cytometry as the detection is limited by particle size in the same order of magnitude wavelength of the laser (about 488 nm). Different machines have varying sensitivities with newer machines able to detect up to 0.3 μm-sized particles.
2. It is subjective and difficult to automate.
3. Centrifugation speeds for sample processing are variable and not standardized.

A variety of flow cytometry markers have been used for detection of MP. These may be divided into primarily those that detect activation status and those that define the cell of origin. Multicolored flow cytometry is a useful approach to define the different sets of MP in circulation. In addition to those listed in table 2, antibodies against CD14, CD3/CD4/CD8 and CD20 are used to detect MP derived from monocytes, and T- and B-lymphocytes, respectively.

Enzyme linked immunoassays/capture assays

In comparison to flow cytometry, antigen capture assays using the enzyme linked immunoabsorbent technique has been less commonly used. The capture of MP into immobilized annexin V or cell specific antibodies have been the major approaches.[59] Solid phase assays have the advantage of detecting MP irrespective of size. However

interference of soluble antigens, variable quality of antibodies used for antigen capture and non-exclusion of exosomes are some of the disadvantages. Table 3 compares Flow cytometry to solid phase assays for MP detection and measurement.

In one of the more commonly used approaches, annexin V is used for capturing MP bearing phosphotidylserine. Biotinylated annexin V are insolubilized onto streptavidin-coated microtitration plates and platelet-free plasma is then added, incubated and washed.[59] Background values obtained with irrelevant IgGs are subtracted from those measured with specific antibodies. The drawback of using Annexin V capture assays is that only a fraction of MP express phosphatidyl serine and most endothelial MP do not express this antigen.

Functional assays (clot based assays)

MP produced as a result of cell activation are likely to express phosphotidyl serine on the cell surface. A novel Factor X-activated clotting time (XACT) has been recently described to measure procoagulant activity of MP in human plasma.[60] Other clot-based assays such as a dilute APTT could potentially be used for determining the procoagulant function of membrane bound phospholipid in plasma samples. The contribution of the soluble fraction of phospholipid in these tests is not entirely clear.

The exact correlation of clot-based tests with various subsets of MP is not well established. It is more than likely that a particular subset, such as platelet derived MP or tissue factor bearing MP, preferentially influences the clotting assay.

Combination of functional and capture assays

The phosphatidylserine content of MP is measured through its ability to promote the activation of prothrombin to thrombin. The blood clotting factor concentrations have been

determined to ensure that this phospholipid is the rate-limiting parameter of the reaction. Immobilized MP are incubated with factor Xa, factor Va, prothrombin and $CaCl_2$ in buffer. Chromogenic substrate for thrombin is then added and linear absorbance changes are recorded at 405 nm using a microtitration plate reader equipped with kinetics software. Results are expressed as nanomolar phosphatidylserine equivalent by reference to a standard curve constructed using liposomes of known concentration, containing phosphatidylserine and phosphatidylcholine in a 1:2 ratio.[59] Commercially available kits have simplified previously in-house based ELISA assays. However, the criterion of Annexin V binding and quantitation by a prothrombinase assay only, selectively measures a subset of MP and can potentially give misleading results.

Quantification of microparticles

MP may be quantified by the following methods in flow cytometry:

1. As a proportion of platelets in the sample.[57]
2. As an absolute number calculated from a known quantity of commercial beads and the dilution factor.[16,61]
3. Expressed in terms of fluorescence compared to standard flourospheres.

When using ELISA techniques, MP may be quantitated with reference to a standard curve created by using liposomes containing a known quantity of the antigen of interest.[8]

EFFECT OF PROCESSING AND STANDARDIZATION OF MICROPARTICLE ANALYSIS

Although a number of publications linking MP to thrombosis have been published in recent years, there has been only a few publications relating to standardization of techniques.[59] The ‘normal’ MP count range in human plasma of healthy subjects is not

established and is easily debatable. This is mainly due to the different processing and analysing techniques being used by various investigators. The main contributing factors to the MP analysis variability are:

1. Preanalytical variables - use of whole blood versus plasma, storage of samples (eg freezing versus immediate processing) and degree of manipulation thought to be acceptable.

2. Analytical variables - as in method employed, such as ELISA versus Flow cytometry.

3. Quantification method - commercial beads versus platelet fraction. ELISA assays have been quantified chromogenically in contrast to flow cytometry where counting beads are used.

4. Type of MP measured - endothelial versus platelet versus leukocyte versus total.

5. The overlap of size range between 'small' platelets and some MP by flow cytometry and the inability of ELISA to separate soluble and membrane bound antigens.

6. Patient group and / or population studied.

There is relative agreement that citrated plasma is the sample of preference.[59] Samples collected in EDTA invariably result in platelet activation and thus are not suitable for analysis of MP and in particular of platelet MP. Although EDTA samples for platelet MP were analyzed by ELISA in one study[59] these have not been compared with another method or alternative anticoagulant. The two preanalytical issues critical to evaluation of MP by flow cytometry or ELISA are the effect of freezing and centrifugation speeds.[62] Although freezing has been reported not to affect MP counts in several studies, there has

not been an extensive evaluation of this step at varying concentrations of MP. In one analysis of endothelial MP, fresh plasma samples were found to be superior to frozen samples.[59] Most other groups that were part of the forum on measuring MP (ISTH, 2004), however, believe that freezing samples does not seem to significantly affect MP counts.

The ranges of MP in circulation have varied depending on the number and speed of centrifugation steps. A wide variety of centrifugation speeds have been employed in an attempt to concentrate or pellet microparticles. It appears that centrifugation at 1500g alone produces a cell free plasma but with significant numbers of platelets. In fact, accurate quantification of MP by flow cytometry in these samples could be technically difficult in view of the some overlap in size between MP and platelets. Additional centrifugation at various speeds has been tried in order to simultaneously remove platelets (platelet poor plasma, PPP) and preserve the MP. In cases where 1500g was the only spin employed, the platelet contamination influencing the range of MP is clearly evident.[62]

Some of the analytical variables for platelet microparticles such as comparison of specific antibodies and linearity have been evaluated.[63] 'Megamix beads' is a novel approach to standardizing of gating of MP by flow cytometry.[64] It uses a mix of a 0.9um and 0.3um sized beads in an attempt to capture all events within the gate set by the beads. One of the problems of using this approach is the lack of linearity in the relationship between the size of beads and forward scatter at that particle size (i.e. at less than 1 micron size). The issue of variability between observers, instruments and laboratories also needs to be addressed.

In 2004, the ISTH developed a subcommittee to report on MP detection and quantification. This subcommittee has since met on an annual basis to update and review the methodology. In spite of this effort there remains a wide variation in the techniques employed to detect MP.

RECENT DEVELOPMENTS IN MICROPARTICLE ASSAYS

Several recent technical advances have been made to improve the detection and measurement of microparticles.

The use of Bio-maleimide as a stain for platelet Microparticles

Bio-maleimide is a cost effective and easy method for MP *detection*[56] and has evoked interest because of its property to attach to biological membranes via cysteine residues in proteins and thiolated oligonucleotides (Thiol-reactive probes excited with visible light, Chapter 2 –thiol reactive probes, Molecular probes. website: www.probes.com; accessed July 2007) and its fluorescent nature enables it to be used in flow cytometry. It is also relatively inexpensive compared to at the price of a standard fluorescently labeled monoclonal antibody. One of the problems with this technique is its inability to detect the cell of origin of MP. Given the wide range of MP counts, predicting the total count may not be entirely useful unless the cellular origins of MP can be determined. However, Bio-maleimide can be used to differentiate cellular material from background 'noise' by flow cytometry.

Newer methods of detection of microparticles

These include:

a. Impedance based flow cytometry for detection of very small MP.[65] This is being developed to overcome the size discrimination limiting factor evident in most standard flow cytometers. It uses impedance to measure size instead of forward scatter and is reportedly more sensitive.

b. Tissue factor dependent procoagulant assay.[66] This assay is designed to capture tissue factor bearing microparticles and measure their procoagulant activity.

c. A proteomics based assay.[67,68] A platform for detection and quantification of a wide variety of circulating antigens (both soluble and membrane bound) in a automated fashion is being developed.

CONCLUSIONS

Circulating microparticles are now widely regarded as transcellular vectors of information, refecting the state of health or disease of the blood cells, endothelium and possibly most organ systems. Although a variety of techniques are currently available for evaluation of these subcellular fragments, there is limited information regarding the relevance of MP measurements in specific clinical states. This is generally due to the high variability and lack of standardisation of MP detection techniques, each with its own advantages and disadvantages. An international consortium is addressing this issue, and the recent introduction of assays that combine functional assessment with measurement of actual numbers of MP may prove more useful in the clinical setting. In the meantime, MP are proving to be multifaceted mediators of blood cell and endovascular changes and are emerging as an important research tool for vascular biology.

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Figure 1. Comparison of sizes of exosomes, microparticles and platelets

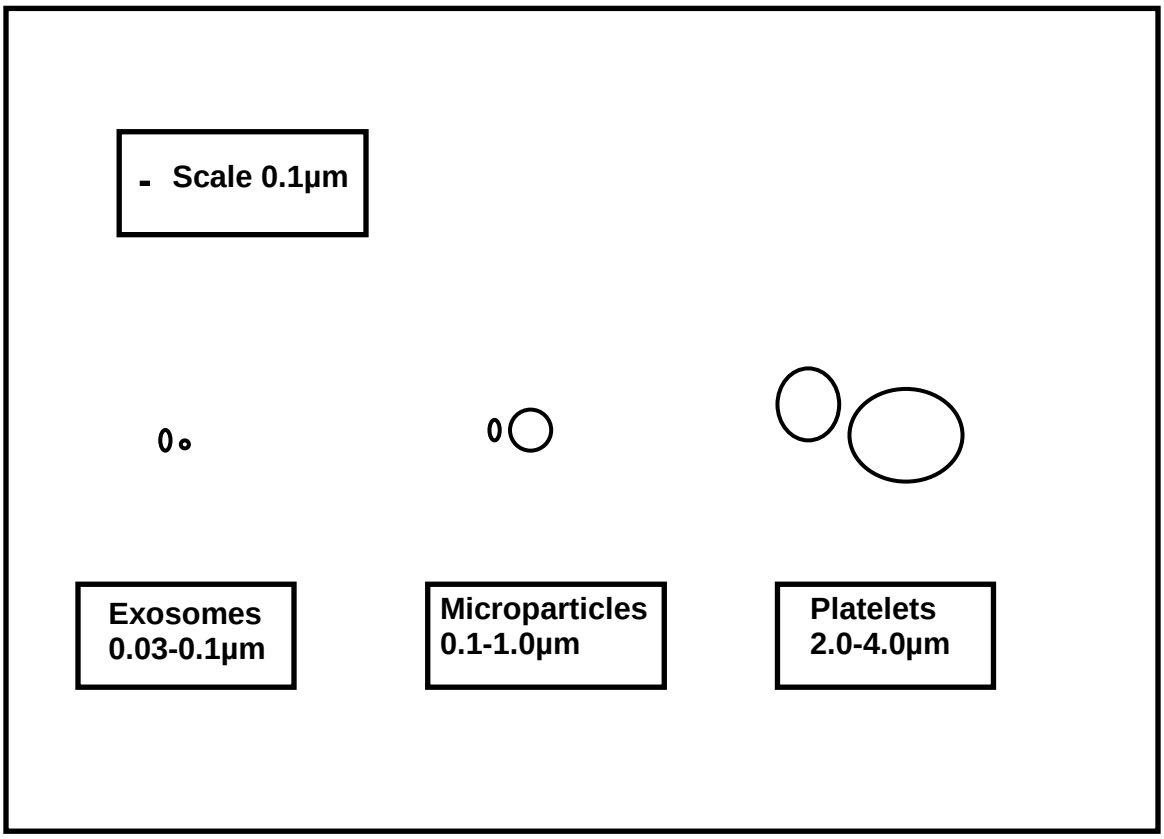

Figure 2. The production of Microparticles and their effects.

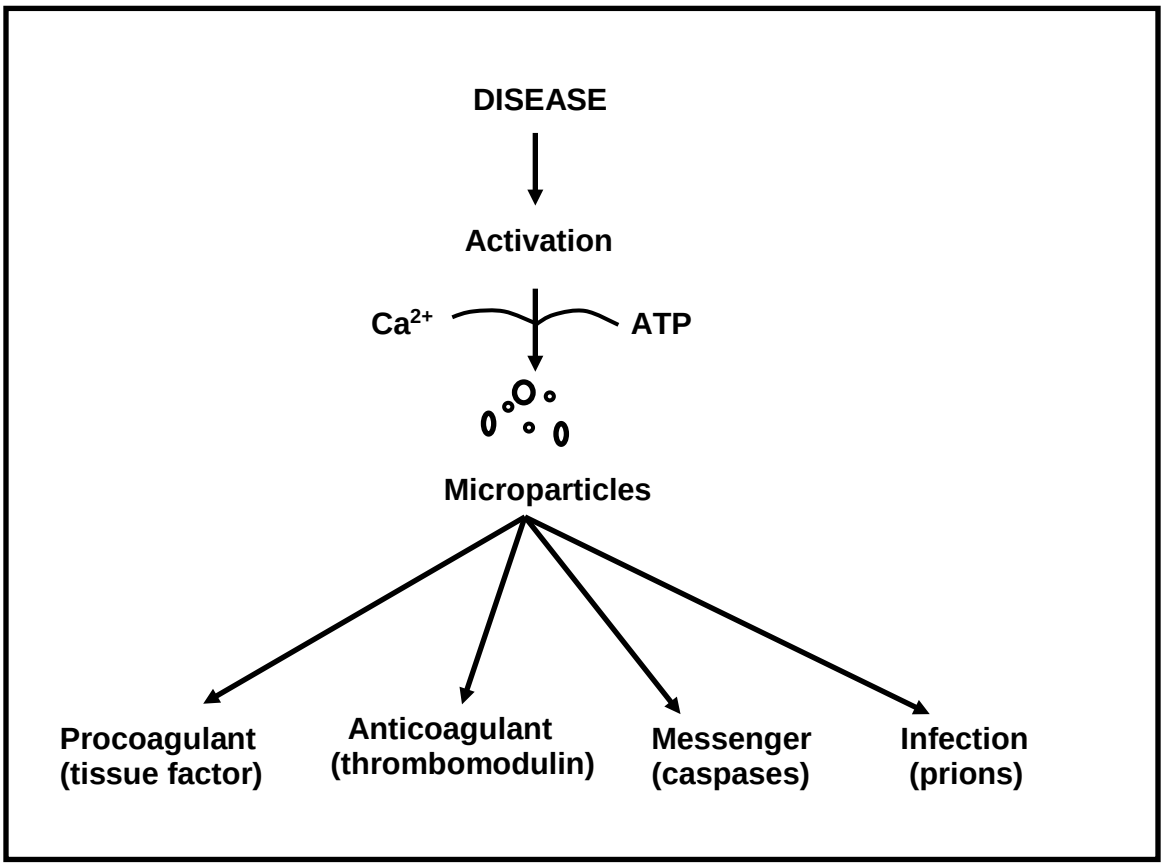

Table 1. Classification of Microparticles

Basis of classification				
Effects	**Coagulant property**	**Cellular origin**	**Function**	**Antigen expression**
Deleterious or Beneficial	Anticoagulant or Procoagulant	Platelet Endothelial Leukocyte Monocyte Malignant	Messenger Adhesin Coagulant Infectious particle	Annexin V Tissue factor P-selectin

Table 2. Different methods for detection of Microparticles: Some highlights.

Authors	Technique	Sample collection tube	Centrifugation	Isolation of MP	Generic MP detection	Antigens for specific cell detection		
						Platelet	Endothelial	Leukocyte
Biro *et al* [22]	Flow cytometry	Citrate	1550*g*, 20 min	18,000 *g*, 30 min	Annexin V	CD62P CD61 CD63	CD31, CD62E or CD144	CD4 CD8
Dignat-. George *et al* [52]	Flow cytometry	Citrate	1500 *g*, 15 min 13,000 *g*, 2 min	None	Annexin V	CD41	CD51, CD144 or CD146	CD45
Freyssin-et *et al.*[53]	Solid phase capture	Citrate	1500 *g*, 15 min 13,000 *g*, 2 min	None	Annexin V, tissue factor	CD62P or GPIba	CD31 or CD62E	CD45
Jimenez *et al.* [54]	Flow cytometry	Citrate	200 *g*, 10 min 1500 *g*, 7 min	None	None	CD41 or CD42b&CD31	CD31+/-CD42 or CD62E	CD45
Nomura *et al* [55]	ELISA	EDTA	1500 *g*, 20 min	None	None	GP IX(capture) CD62P, CD40L	None	None
Enjeti *et al* [56]	Flow cytometry	Citrate	1500 g, 30min 13,000 g, 2min	None	Maleimide	CD41	CD62e	CD45
Dale *et al*[57]	Flow cytometry	Citrate	Platelet rich plasma	Activation of coated platelets	maleimide	GPIIb/IIIa	None	None
Shet *et al* [37]	Flow cytometry	Citrate	13,000 *g*, 10 min	100,000 *g* 60 min	Annexin V	CD41a	CD144	None

Table 3. Comparison of flow cytometry and ELISA techniques to detect and measure MP

Parameter	Solid Phase capture assays	Flow Cytometry based assays
Antigen detection	soluble and membrane bound one at a time expression and/or function	membrane bound only multiple at a time expression only
Particle Size	No lower limit	limited by light wavelength used (probably around 0.2-0.3 um)
Sensitivity	high Sufficiently sensitive to pick up weak antigen expression	low Limited ability to detect weakly expressed antigens such as endothelial antigens
Automation	Although more labour intensive if doing an in-house assay, easier to automate once established or if using a kit	More difficult to automate
Cost /100 tests	$400-1000 depending on assay	$200-500 depending on antibody

Aim 1:

To evaluate different techniques of microvesicle detection and measurement by correlating MV analysis by flow cytometry, functional ELISA, XaCT test, thrombin generation and nanotracking in a cohort of normal healthy subjects.

PAPER 1: Enjeti AK, Ariyarajah A, D'Crus A, Seldon M, Lincz LF. Correlative analysis of nanoparticle tracking, flow cytometric and functional measurements for circulating microvesicles in normal subjects. *Thromb Res*. 2016;145:18-23.

Key learning and reflections

Certain parameters such as PS or TF expression on MV by flow cytometry correlates with thrombin generation assays. Quantity and function are two facets of MV evaluation.

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Full Length Article

Correlative analysis of nanoparticle tracking, flow cytometric and functional measurements for circulating microvesicles in normal subjects☆

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ABSTRACT

Introduction: Circulating microvesicles (MV) can be analysed using a number of different techniques. The aim of this study was to evaluate the correlation between functional procoagulant based assays including thrombin generation, factor Xa activation test (XaCT), and phosphatidylserine factor Xa-activity by ELISA with optical MV enumeration by flow cytometry and nanoparticle tracking analysis.
Methods: Citrated blood samples were collected from 60 healthy volunteer blood donors after informed consent. Platelet free plasma was prepared using a standardized published protocol. MV subsets were enumerated by flow cytometry (BDFACS Canto) after staining with specific antibodies for platelets (CD41), endothelial cells (CD105), red cells (CD235) monocytes (CD14), tissue factor (CD142) and for phosphatidylserine expression by binding to annexin V. A standardized protocol using counting beads was employed. Nanotracking analysis was performed on both scatter and fluorescent settings after MV staining with quantum dot stain, Qdot 655. Procoagulant function was assessed by the XaCT assay on an automated coagulation analyser and by thrombin generation assay measuring endogenous thrombin potential (ETP), lagtime, peak (PEAK) and time to peak (ttPEAK) using a Calibrated Automated Thrombogram (CAT). The statistical analysis was carried out with Statistica 12 software using non-parametric tests (Spearman rank order correlations, with significance set at $p < 0.05$).
Results: In normal healthy subjects, thrombin generation parameters correlated with levels of MV measured by flow cytometry. ETP, lagtime, ttPEAK and PEAK correlated with MV expressing phosphatidylserine (r_s, Spearman rank order correlation was 0.29, 0.40, 0.31 and 0.34 respectively, $p < 0.05$), and MV expressing tissue factor (r_s was 0.29, 0.40, 0.31 and 0.34 respectively, $p < 0.05$), whilst red cell derived MV correlated with lagtime, ttPEAK and PEAK (r_s, was 0.35,0.30 and 0.3, respectively, $p < 0.05$). Lagtime and ttPEAK negatively correlated with the clot based XaCT test (r_s, was −0.34 and −0.30 respectively, $p < 0.05$) and positively correlated with the ELISA MP-activity assay ($r_s = 0.42$ for both, $p < 0.05$). In addition, endothelial MV levels weakly correlated with white cell counts ($r_s = 0.27$, $p < 0.05$).
Conclusions: Thrombin generation and flow cytometry for phosphatidylserine or tissue factor expressing MV correlate well as markers for procoagulant activity. A combination of optical or non-optical enumeration as well as functional methods may be required for a complete profiling of circulating MV.

1. Introduction

Circulating microvesicles (MV) are currently a focus of intense research, having been implicated in both physiological and pathophysiological processes [1]. With specific reference to coagulation, they are now deemed to play a role in both normal and abnormal haemostasis [2]. However, the accurate identification, characterisation and enumeration of such small particles remain a challenge. Although a number of

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studies have evaluated the pre-analytical variables that can affect MV measurements, very few have addressed the correlation between functional assays and those that focus on enumeration and/or identifying the cell of origin.

The terminology for small circulating, cell derived particles, in the micron or submicron range, namely 'microparticles' (designated as MP) or 'microvesicles' has been used interchangeably in the literature. In contrast, the term 'exosomes' is often reserved for vesicles that are actively secreted by cells and are <50 nm in size [3–5]. For the purpose of this study, we have used the term 'microvesicle' to designate any cell derived anucleate particle in circulation that is 1000 nm or less in size.

Microvesicles can be measured and analysed in a number of different ways. Flow cytometry is a popular and widely used tool due to the ease of the technique and advantage of being able to identify expression of particular antigens that may reveal the cell of origin [6,7]. The deficiencies of flow cytometry include pre-analytical, as well as gating variables, in addition to its limitations in detecting MV < 200 nm in size. Nanotracking analysis (NTA), another optical approach, is capable of accurately measuring these small MV – challenges with this technique include reduced sensitivity in measuring larger particles in a polydisperse sample [8,9].

A variety of functional tests are utilized to evaluate MV, ranging from annexin V capture based ELISA to clot based assays and thrombin generation. Whilst the ELISA measures the factor Xa activity of phosphatidylserine (PS) expressing MV, the clot based and thrombin generation assays measure the contribution of MV to global haemostasis. The functional assays are capable of investigating the ability of the MV to contribute to the process of coagulation, in contrast to optical enumeration assays such as flow cytometry or NTA.

This study was undertaken to evaluate the correlation between functional procoagulant activity of MV with optical measurements using flow cytometry and nanotracking analysis. To date, no articles have been published looking at the correlation between nanotracking measurements and flow cytometry in conjunction with functional assays, in normal healthy subjects.

2. Methods

2.1. Subject characteristics

Blood samples were collected from 60 healthy volunteer blood donors, after receiving informed consent. The average age was 38 ± 16.3 years. The average BMI was 26.1 ± 3.5 kg/m^2. There were 30 males and 30 females in the cohort.

2.2. Sample preparation

Peripheral blood was collected in 0.109 M tri-sodium citrate. Platelet-free plasma was obtained by double centrifugation of whole blood for 15 min at 2500 ×*g*. All samples were processed within 2 h of collection, and aliquots were stored at −80 °C until analysis. Samples were then thawed at 37 °C within 15 min prior to testing. All samples were treated in a similar fashion and thawing was undertaken once, just before testing. None of the samples were refrozen, as different aliquots from the same subject were used where necessary.

2.3. Flow cytomtery

A 10 μl aliquot of platelet-free plasma was incubated (in the dark) at room temperature for 15 min with combinations of CD41a-PE (Clone HIP8, BD Biosciences, CA, USA), CD235a-APC (Clone GA-R2, BD Biosciences, CA, USA), CD105-PE (Clone IG2, Beckman Coulter, Marseille Cedex, France), and CD14-PE (Clone TUK 4, Miltenyi Biotec, CA, USA) and TF-FITC (CD142, Clone VD8, American diagnostics Inc., CT, USA) or appropriate isotype controls in a final volume of 100 μl of PBS. For experiments with annexin V-APC (eBioscience, CA, USA) the incubation was done in a total of 50 μl of binding buffer. The sample was then diluted to 400 μl with filtered PBS or 450 μl of calcium rich buffer for annexin V binding experiments. A known number of 10 μm enumeration beads (CountBright beads, Molecular Probes, Life Technologies, Oregon, USA) were added prior to analysis. The flow cytometer used in these experiments was standardized during the 'ISTH workshop for standardization of flow cytometry for Microparticles' as a participating site [10]. The gating was set as per recommended strategies using Megamix beads (Biocytex, Marseille, France) on a BDFACS Canto instrument (BD, New Jersey, USA). The data was collected and analysed using FACSDiva software.

2.4. Functional coagulation based studies

The factor Xa activation test (XaCT) is a clot based assay which detects procoagulant activity of microparticles/microvesicles, based on the ability of vesicles to generate Xa, using the commercially available XaCT test kit (Haematex, Australia) [11]. It was performed in duplicate according to the manufacturer's instructions on an automated BCS coagulation analyser (Dade Behring, Marburg, Germany). A dilution of plasma calibrator (provided by the manufacturer) generated a standard curve from which the individual sample results were read by the instrument's software expressed in ng/ml.

2.5. Functional ELISA based analysis (Zymuphen phosphatidylserine Xa-activity)

The functional assay for the measurement of MV procoagulant activity in plasma was performed using the Zymuphen PS Xa-Activity ELISA kit (Hyphen Biomed, Neuville-sur-Oise, France) according to the manufacturer's instructions. Essentially, MV in the sample were allowed to bind to the annexin V coated on the surface of the microplate wells. Washing process removed unbound particles and prothrombin added along with factor Xa- factor Va in the presence of calcium.

The production of thrombin was measured via cleavage of a chromogenic thrombin substrate producing absorbance at 405 nm and results derived from a standard curve of known MV concentration expressed in nM phosphatidylserine (PS) equivalent. Thrombin generation is directly related to the phospholipid concentration in the plasma. All samples were analysed in duplicate. Only replicates that were at least 85% concordant were used in the final analysis.

2.6. Calibrated Automated Thrombogram measurements

Thrombin generation experiments were carried out as per the manufacturer's instructions on the Calibrated Automated Thrombogram (CAT) and data analysed on the Thrombinoscope software version 3.0029 (Thrombinoscope, Stago Group, Maastricht, The Netherlands). In summary, 80 μl of sample was incubated with 20 μl of calibrator or 20 μl of specific MP-reagent (Thrombinoscope, Maastricht, The Netherlands). The MP-reagent is reportedly sensitive to tissue factor bearing microparticles/microvesicles [12]. The CAT automatically dispensed the prepared fluorescent substrate and buffer. The parameters evaluated for measuring thrombin generation included endogenous thrombin potential (ETP), lagtime, the peak (PEAK) and time to peak (ttPEAK).

2.7. Nanoparticle tracking analysis (NTA)

The enumeration of MV by nanotracking was undertaken on a Nanosight NS500 instrument (Malvern instruments, Malvern, United Kingdom). The scatter was undertaken on plasma samples and the fluorescence recorded after incubation with a quantum dot, Qdot 655 stain (Qtracker® 655 cell labelling kit, Life Technologies/ThermoFischer Scientific, MA, USA) in a dilution of 1:100 with PBS as per the manufacturer's instructions. The scatter settings and fluorescent capture settings, including camera, focus and gain, were optimised so that

particle tracks were clearly visible. Further adjustments to dilutions were undertaken, where the capture was suboptimal (i.e. event capture rate either <20 or >200 tracks). The fluorescence events were detected using a violet laser and were captured on the instrument's software for analysis. The dilutions were optimised (for both fluorescence and scatter measurements) so that at least 100 completed tracks were recorded. Measurements were taken in triplicate and analysed using Nanosight software (version 2.3 and 3.1). The captured video data was analysed for total events, peak size, events <200 nm, 200 to 400 nm and >400 nm.

2.8. Ethical and statistical considerations

The study was carried out in accordance with the National Statement on Ethical Conduct in Research Involving Humans (Australia). It was approved by the Hunter New England Area Research and the University of Newcastle Ethics Committees. A written informed consent was obtained from each participant on this study.

The statistical analysis was carried out by Statistica 12 software (StatSoft Inc., Dell Software, OK USA) using non-parametric tests (Spearman rank order correlations, r_s) and a p-value <0.05 was considered statistically significant. A further analysis for significance at $p < 0.001$ was also undertaken to correct for multiple hypotheses testing.

3. Results

3.1. Baseline blood counts

All participants' blood results were within normal range - the mean (±standard deviation) haemoglobin was 128.5 ± 10.5 g/l, white cell count was 6.0 ± 1.3 × 10^9/l and platelets 213.3 ± 48.2 × 10^9/l (Table 1).

3.2. Flow cytometry

The mean and median values for circulating MV of different cellular origin are shown in the Table 1. The predominant MV in circulation were platelet (CD41) derived followed by those of red cell (CD235) and endothelial cell origin (CD105) with all flow cytometric measurements showing a wide range. Phosphatidylserine (by annexin V binding) and tissue factor expression were observed in a high proportion of MV. The MV measurements by flow cytometry were not normally distributed and varied widely.

3.3. Functional assays

The results of the three functional assays (CAT, clot based XaCT, and PS capture ELISA) are shown in Table 2. The measurements for ETP, the peak and XaCT tests had a wide range but with a relatively normal distribution.

Table 1
Full blood count and flow cytometry characteristics for the healthy cohort of 60 subjects.

Variable	Mean	Median	Range	Lower quartile	Upper quartile
Hb (g/l)	128.47	129.50	110–148	119.00	137.00
WCC (×10^9/l)	6.09	5.90	3.6–9.7	5.05	6.85
PLTS (×10^9/l)	213.32	209.50	111–339	174.50	246.50
CD41 (MV/μl)	5104.32	1408.12	78.43–67,555.56	21.81	186.44
CD235 (MV/μl)	3964.34	1488.05	0.0–42,375.93	10.38	100.00
CD105 (MV/μl)	3864.36	2861.28	0.0–43,708	6.0	75.00
CD14 (MV/μl)	53.32	17.04	0.21–558.28	6.29	46.28
AnnV (MV/μl)	6668.17	601.71	0.0–72,338.24	9.00	108.79
TF (MV/μl)	3878.30	1408.69	263.24–36,824.24	13.32	89.93

TF, tissue factor. AnnV, annexin V.

Table 2
Functional MV assessment by thrombin generation assays, ELISA PS Xa-activity and XaCT tests.

Variable	Mean	Median	Range	Lower Quartile	Upper Quartile
ETP (RFU/min)	1879.38	1864.50	750.0–3253.0	1409.50	2320.00
Lagtime (min)	4.86	4.88	3.50–7.00	4.20	5.33
PEAK (nM)	420.90	424.22	178.26–850.70	339.73	482.03
ttPEAK (min)	6.86	6.67	5.00–9.10	6.00	7.67
XACT (ng/ml)	936.11	909.70	448.6–2025.0	686.80	1115.40
ELISA (nM PS Xa-activity)	9.45	7.00	5.00–19.00	6.00	14.00

RFU, relative fluorescent units. ETP, endogenous thrombin potential. ttPEAK, time to peak.

3.4. Nanoparticle tracking analysis

The median number of MV was 9.10 × 10^7/μl on scatter mode and 37 × 10^7/μl as measured by fluorescence. The majority of vesicles (i.e. peak size) measured 61 nm in scatter mode and 110 nm in fluorescent mode as shown in Table 3. The distribution of various vesicle sizes by both modes of detection is also shown.

3.5. Correlations

3.5.1. Full blood count results: Endothelial MV correlate with white cell counts

It was observed that endothelial MV correlated with white cell counts ($r_s = 0.27$, $p < 0.05$), however, the platelet, red cell or monocyte derived MV did not correlate with any of the blood counts. The full blood counts did not correlate with thrombin generation assays, ELISA or NTA results.

3.5.2. Thrombin generation assays correlate with phosphatidylserine and tissue factor expressing MV by flow cytometry

All parameters of thrombin generation assessment correlated with the key flow cytometry markers of procoagulant activity i.e. phosphatidylserine or tissue factor expressing MV (Table 4). PS expression (by annexin V binding) remained significantly correlated after correction for multiple hypotheses testing ($p < 0.001$). The thrombin generation parameters of lagtime, peak and time to peak also correlated with red cell MV. None of the other flow cytometry markers, i.e. MV expressing CD105, CD41 or CD14, correlated with thrombin generation parameters.

Table 3
MV nanoparticle tracking analysis results by scatter and fluorescent mode.

Variable	Mean × 10^7/μl	Median × 10^7/μl	Minimum × 10^7/μl	Maximum × 10^7/μl	Lower quartile	Upper quartile
Scatter	33.6	9.10	0.060	670.00	4.27	18.00
S peak[a]	116.9	61.00	27.00	427.00	50.00	163.00
S < 200 nm	18.6	2.60	0.00	369.00	0.34	6.53
S 200–400 nm	12.0	3.46	0.03	282.00	1.45	8.30
S > 400 nm	2.62	0.87	0.00	18.10	0.31	3.80
Fluorescent	45.78	37.00	13.00	155.00	26.00	51.00
F peak[a]	139.17	110.00	54.00	366.00	88.00	166.00
F < 200 nm	0.77	0.60	0.00	4.00	0.27	1.00
F 200–400 nm	0.62	0.26	0.00	14.00	0.05	0.58
F > 400 nm	78.18	78.00	32.00	133.00	65.50	90.50

S, scatter. F, fluorescence.
[a] A measure of the number of particles at peak size by scatter or fluorescence.

Table 4
Correlation of thrombin generation assays and flow cytometry (Spearman's rank order correlations, numbers in bold are statistically significant at $p < 0.05$).

Variable	CD105	CD41	CD235	CD14	AnnV	TF
ETP	−0.02	0.01	0.24	−0.06	**0.29**	**0.30**
Lagtime	−0.16	0.15	**0.35**	−0.05	**0.40**[a]	**0.32**
PEAK	−0.03	−0.05	**0.30**	−0.04	**0.31**	**0.34**
ttPEAK	−0.15	0.15	**0.30**	−0.04	**0.34**	**0.26**

ETP, endogenous thrombin potential. ttPEAK, time to peak.
[a] Correlations reached significance at $p < 0.001$.

3.5.3. Thrombin generation parameters (lagtime and ttPEAK) correlate with functional studies of XaCT and ELISA but not with MV measured by NTA

Thrombin generation parameters of lagtime and ttPEAK correlated negatively with XaCT but positively with ELISA MP-activity. There was no correlation between thrombin generation assays and measurement of MV by scatter or fluorescent NTA as seen in Table 5.

3.5.4. Endothelial, platelet and PS expressing MV by flow cytometry correlate with MV measured by NTA

The endothelial (CD105) MV measurement by flow cytometry correlated with total MV measured by scatter NTA. The CD105 and CD41 correlated with scatter peaks with the latter showing a negative correlation. Table 6 shows that NTA fluorescent peak correlated with total PS expressing MV and MV between sizes of 200-400 nm on fluorescence, as shown in Table 6.

4. Discussion

This study elucidates the relationship between functional studies for evaluating MV as compared to flow cytometry and nanotracking results, which focus on microvesicle enumeration. This is the first study that combines flow cytometry, functional assays, as well as NTA to characterize MV in a cohort of healthy subjects.

Several different approaches to detection and measurement of MV have evolved in recent years. In this study, we opted to correlate the most widely used and easily available techniques, which included a combination of optical and functional assays. The optical techniques used were flow cytometry and NTA. Flow cytometry enumerates by gating strategies using a mix of standard beads and counting beads added to the sample during analysis. The NTA uses Brownian motion of particles and tracks them for enumeration. The functional assays used were thrombin generation assessment (on CAT), Zymuphen ELISA based Xa activity and the XaCT clot based assay. The CAT estimates the thrombin generation parameters related to phospholipid vesicles in the sample whilst the XaCT test evaluates the contribution of the all membrane-derived phospholipids, from microvesicle surface, to factor Xa activity. Although the ELISA also estimated factor Xa activity, this is limited to the captured PS expressing MV. Given that this is an exploratory study, a p value of <0.05 was considered significant in our correlative analysis to avoid a type II error.

Table 5
Correlation of thrombin generation assays with XaCT (clot based), ELISA (Zymuphen PS Xa-activity) and NTA (Spearman's rank order correlations, numbers in bold are statistically significant at $p < 0.05$).

Variable	XaCT Clot based	ELISA MP-activity	Scatter NTA	Fluorescence NTA
ETP	−0.05	0.13	0.08	0.02
Lagtime	**−0.34**	**0.42**	−0.14	0.08
PEAK	−0.09	0.15	0.05	0.02
ttPEAK	**−0.30**	**0.42**	−0.12	0.02

ETP, endogenous thrombin potential. ttPEAK, time to peak.

Table 6
Correlation of NTA and flow cytometry (Spearman's rank order correlations, numbers in bold are statistically significant at $p < 0.05$).

Variable	CD41	CD105	CD235	TF	AnnV	CD14
Scatter	0.06	**0.30**	−0.10	−0.19	0.06	0.09
S peak[a]	**−0.33**	**0.27**	0.03	−0.06	0.20	0.12
S < 200 nm	0.23	0.05	−0.12	−0.17	−0.01	0.06
S 200–400 nm	−0.01	**0.31**	−0.21	**−0.26**	0.02	0.17
S > 400 nm	−0.14	0.22	0.054	−0.15	0.07	0.05
Fluorescence	0.08	0.03	−0.05	0.14	0.16	−0.01
F peak[a]	0.10	0.02	0.12	0.17	**0.26**	−0.04
F < 200 nm	0.08	−0.04	0.02	0.08	0.13	0.01
F 200–400 nm	−0.20	**0.39**	0.03	−0.13	0.03	0.04
F > 400 nm	0.09	−0.24	−0.03	0.13	−0.02	−0.08

S, scatter. F, fluorescence.
[a] A measure of the number of particles at peak size by scatter or fluorescence.

The key observations in our study were that in normal subjects, thrombin generation parameters correlated positively with PS and TF expressing MV by flow cytometry. The levels of red cell (CD235) MV by flow cytometry also correlated positively with lagtime, ttPEAK and the peak for thrombin generation. Lag time and time to peak negatively correlated with the XaCT test and positively correlated with the Zymuphen ELISA assay. In addition, endothelial MV appeared to correlate with white cell counts.

It was observed that PS and TF expression on MV by flow cytometry correlated with all the thrombin generation parameters including ETP, ttPEAK and the peak. Being a negatively charged phospholipid, PS has the ability to act as a catalytic site for activated factors X and V [13]. Moreover, microvesicles expressing PS have been associated with prothrombotic states such as anti-phospholipid antibody syndrome [14]. Tissue factor MV have been associated with increased risk of venous thromboembolism (VTE) particularly in certain pathological states such as haemophilia as well as cancer related VTE [15–18]. In animal models, haemopoietic cell derived TF has been established to contribute to formation of thrombus indicating the significance of TF MV which is critical to thrombosis [19]. The TF MV have also been described to be circulating in normal healthy subjects and flow cytometric estimation of TF MV in circulation appears to indicate functional activity in samples from patients with acute coronary syndromes [20,21]. The lag time also positively correlated with both PS and TF expression by flow. Lagtime reflects the initiation phase of thrombin generation and it is intriguing why TF expressing MV positively correlated with this parameter. The explanation may lie in the limitation of flow cytometry, as it has been shown that TF MV which are associated with procoagulant activity are usually smaller than 200 nm, which is below the detection limit for the flow cytometer [22].

In our study, although the platelet derived MV as measured by flow cytometry averaged the highest amongst the various types, the endothelial and red cell derived MV were present in significant numbers and in fact, with higher median counts. The PS and TF expressing MV, which represent a variety of cell derived MV, are key contributors to MV associated procoagulant activity. Both red cell and endothelial MV have been implicated as key contributors to haemostasis. Red cell MV in stored blood bags are reported to have thrombogenic potential [23–25]. Endothelial MV have been shown to be important in mediating thrombosis particularly in conditions where the endothelium is activated such as anti-phospholipid antibody syndromes [26]. In this study, although red cell MV correlated with thrombin generation parameters as expected, the endothelial MP did not. This may be due to the fact that this cohort was composed only of normal subjects, whereas most studies reporting contribution of endothelial MV have been in pathological states involving endothelial activation [27–29]. The investigation of additional specific endothelial activation markers, such as expression of CD106 (VCAM, vascular cell adhesion molecule) and CD54 (ICAM,

intercellular cell adhesion molecule) in addition to CD105 could clarify this issue and is a limitation of the present study.

Thrombin generation assays on MV have been reported to correlate well with levels measured by transmission electron microscopy for MV generated from cancer cell lines [22]. Thrombin generation potential in MV also seem to correlate with recurrence in patients with thrombosis [30]. Considering thrombin generation related measurements as the critical parameters for measuring procoagulant activity of MV, it was observed in our study that lagtime and ttPEAK correlated with XaCT test as well as factor Xa-activity by ELISA (Zymuphen PS Xa-activity). The negative correlation with the XaCT test can be explained by the fact that higher phospholipid based procoagulant Xa generation activity is likely to also shorten the lagtime as well as ttPEAK.

Compared to the XaCT test, the factor Xa-activity by ELISA is a more complex test, which relies on specific capture of PS expressing MV only and subsequent factor Xa generation. The XaCT test on the other hand depends on all membrane vesicle phospholipids in the sample, not merely the PS expressing MV. The proportion of PS expressing MV as part of all phospholipids is not quantifiable by this method and may explain the discrepant results between XaCT and ELISA. The PS expressing MV derived from agonist stimulated platelets are reported to contribute to a significant proportion of procoagulant activity using an in vitro model [31]. The positive correlation between lagtime and ttPEAK by CAT and the ELISA PS factor Xa-activity seen herein, indicates that lagtime and ttPEAK increase with increased proportion of MV binding to annexin V. This suggests that the relative numbers or nature of contribution of PS expressing or non-expressing MV to procoagulant activity may be different in a healthy cohort compared to those from agonist-stimulated platelets. Whilst thrombin generation assays may be more comprehensive for procoagulant activity, neither thrombin generation or clot based assays distinguish between the relative contributions of MV derived from different cells. Given the heterogeneous nature of human plasma, the relative contributions are difficult to tease out with the current range of methodology.

This study did not show correlation of platelet MV by flow cytometry with any of the functional assays. Most flow cytometry based studies have focussed on the procoagulant properties of platelet MV. It has been observed, whilst platelet-derived MV contribute to platelet derived procoagulant activity, platelet derived soluble factors also contribute significantly [32]. Platelet MV may also mediate haemostasis via other mechanisms such as neutrophil activation and aggregation [33]. In a previous study by Ayers et al., it was shown that lactadherin positive MV correlated with thrombin generation parameters and weakly with ELISA (Zymuphen PS Xa-activity) [34]. They also observed a negative correlation between their coagulation assay and flow cytometry. Their study used samples from a combination of normal as well as patients with obstructive sleep apnoea. This is different to our study where we have studied a larger cohort of normal subjects only [34]. In another study comparing MV derived from platelets and monocytes, it was shown that monocyte MV had higher TF and prothrombinase activity compared to platelet MV [35]. Given the normal healthy cohort, only low levels of monocyte MV were seen in this study and did not specifically correlate with the functional studies. The evidence for correlation of circulating platelet derived microvesicles and phospholipid dependent procoagulant activity is conflicting, with at least one other study demonstrating a lack of correlation [31]. Dedicated and MV specific flow cytometers may improve the measurement by light scatter techniques [4].

In a different centrifugation approach by Poncelet et al., they observed that a 13,000 × *g* centrifugation step is able to isolate particles which showed linear correlation between enumeration of platelet MV by flow cytometry and several functional studies including thrombin generation [36]. This was also observed in another study where centrifugation clearly influenced platelet derived MV levels in plasma [37]. The alternative centrifugation step is therefore likely to preferentially isolate procoagulant platelet derived MV. The samples in our study were centrifuged using a standard protocol and the above variation in centrifugation step could not be replicated due to limitations in the sample volume.

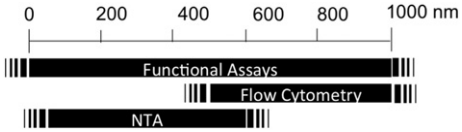

Fig. 1. Overlap between functional and optical methods to evaluate MV and size range for the various methods.

The observation that flow cytometry enumeration does not always correlate with other novel optical methods such as NTA is well established [3]. It was observed in our study that PS expressing MV correlated with particles between 200 and 400 nm on NTA analysis on the fluorescent mode. The significance of this is not clear but it may be that PS expressing MV is concentrated in a certain size range. The ability of flow cytometry to detect particles of varying sizes in a complex sample such as human plasma may be limited not only by the lower limit of size detection but also by swarm effect [5]. The flow cytometric techniques for measuring MV are evolving and although this study followed the recommended ISTH protocol, a MV specific flow cytometry instrument was not used.

The correlation of functional studies with NTA, however, is not clearly established nor widely reported. Certainly, using thrombin generation and correlating it with transmission electron microscopy, it was observed that although three quarters of TF activity from cancer cell lines was associated with particles < 0.1 μm or 100 nm, only a third of the total MV are <100 nm [22]. Another quarter of procoagulant activity is associated with these cancer cell line derived TF bearing particles, which are larger than 100 nm [22].

We did not observe a correlation between measurements by NTA and functional assays, reasons are not obvious but possibilities include that this was a normal cohort of subjects and PS or TF bearing MV may be differently distributed amongst normal subjects compared to pathological samples. Fluorescence based NTA has potential to provide information on MV of small sizes in real time experiments and to specifically identify particles which have a biological origin. Quantum dots have been traditionally used for applications requiring high signal to noise ratio with peak emission at 605 nm and a diameter of around 15 nm, they are small enough to fit into the vesicles as small as 24 nm [38]. The technique is therefore useful to identify vesicles in the size range of 100 nm and smaller. We anticipated that Qdot staining may identify a subset of MV which may better correlate with functional studies, but this was not the case. In contrast to flow cytometry, NTA is unable to determine the cell of origin and this is a major deficiency of the latter approach. Non-optical methods such as atomic force and transmission electron microscopy are less widely available, but may also provide useful quantitative measurements. Resistive pulse sensing is another non-optical technique that can be used for measuring MV-. This technique is advantageous in measuring homogenous samples, but may be challenging to use for heterogeneous samples [4]. This study is limited by not using a non-optical technique to confirm the findings observed from optical or functional assay.

In conclusion, PS or TF expression on MV by flow cytometry appears to correlate with thrombin generation assays. Particle enumeration by NTA according to size does not correlate with procoagulant activity. Therefore, flow cytometric detection of PS and TF MV has potential to be used as a surrogate marker for procoagulant activity. Enumeration and function represent separate facets for evaluation of MV, with flow cytometry and NTA able to define larger and smaller particles respectively, whilst functional analysis is across the entire range of vesicle sizes (Fig. 1). This study reinforces the widely held opinion that a variety of optical, non-optical and functional approaches may need to be undertaken for complete profiling of MV in circulation.

Conflict of interest

None.

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3.8 Improvements in optical and non-optical MV assays

Several recent technical advances have been made to improve the detection and measurement of MV. This includes the following developments:

3.8.1 Stains/Antibodies

Biomaleimide has evoked interest because of its property to attach to biological membranes via cysteine residues in proteins and thiolated oligonucleotides and its fluorescent nature enables it to be used in flow cytometry.[47] One of the problems with this technique is its inability to detect the cell of origin of MV. Predicting the total count per se using a generic marker may not be useful unless the cellular origins of MV can also be determined. [82] PHK67 and annexin V are other commonly used stains used as generic markers for MV. Although annexin V does not stain all MV, greater than 80% of MV in human plasma are annexin V positive and it is a widely used marker in flow cytometry.[83]

3.8.2 Flow cytometers

Impedance based flow cytometry and nanoFACS for detection of very small MV is a recent innovation. These were developed to overcome the size discrimination limiting factor for most standard flow cytometers. They use the principle of impedance to measure size instead of forward scatter and are reportedly more sensitive. [43]

3.8.3 New software modules

Nanoview modules added to traditional flow cytometers enables capture and analysis below usual resolution by a custom design which maximizes event-generating signals and minimises the noise in the forward scatter.[84] This enables detection of MV below the size limit imposed by the wavelength of light used in the standard flow cytometer.

3.8.4 Functional and other approaches

Tissue factor dependent procoagulant assay is designed to capture tissue factor bearing microvesicles and measure their procoagulant activity.[48]A proteomics based platform for detection and quantization of a wide variety of circulating antigens (both soluble and membrane bound) in an automated fashion is being developed.[49]

3.8.5 Other innovative technologies for measuring MV

These technologies can also be broadly classified as optical and non-optical. The former includes techniques based on light scatter (dynamic light scattering and nanoparticle tracking analysis) or fluorescence. Non-optical modes include nano-pore sizing, transmission electron microscopy and atomic force microscopy. [7,11,64]

Atomic force microscopy(AFM) and transmission electron microscopy are useful to study single vesicles. These can assist in probing the heterogeneity of MV/EV as these techniques have high sensitivity and resolution.[85] Using AFM and infrared spectroscopy, it is also possible to investigate the molecular composition of vesicles. [86] This technique is also label free and requires a very small sample volume.[85] However, it does require expensive equipment and specialized operational capacity.

Tunable resistive pulse sensing (TRPS) technology is a non-optical technique based on passage of particles through a nanoscale pore. As a particle moves across a nanopore, a discrete blockade signal event is generated.[87] The magnitude of the measured blockade signal is a key indicator of the volume or the size of the particle that passed through the pore as seen in figure 3(b). Particle by particle detection occurs enabling size distribution within a sample and is suitable for samples with particles of variable size. [87]

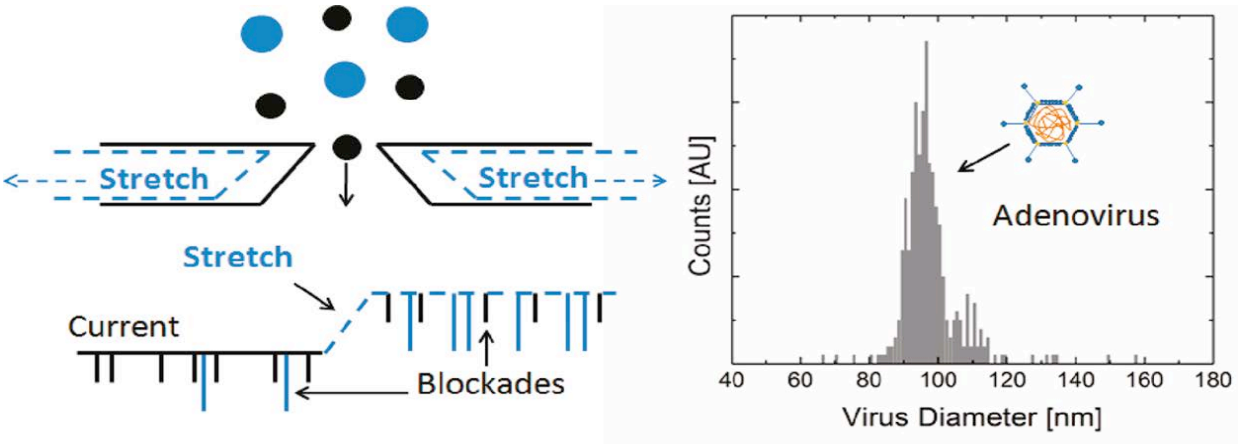

Figure 3(b). Mechanism of a nano-pore sizing method for a 100nm virus particle. Reproduced from reference.[87]

Nanoparticle tracking analysis (NTA) is an optical analysis based on tracking the Brownian motion of nanoparticles in liquid suspension.[88] Subsequent application of a mathematical equation allows the determination of particle size. A software program provides a computational map of particle size and number as shown in figure 3(c).[89]

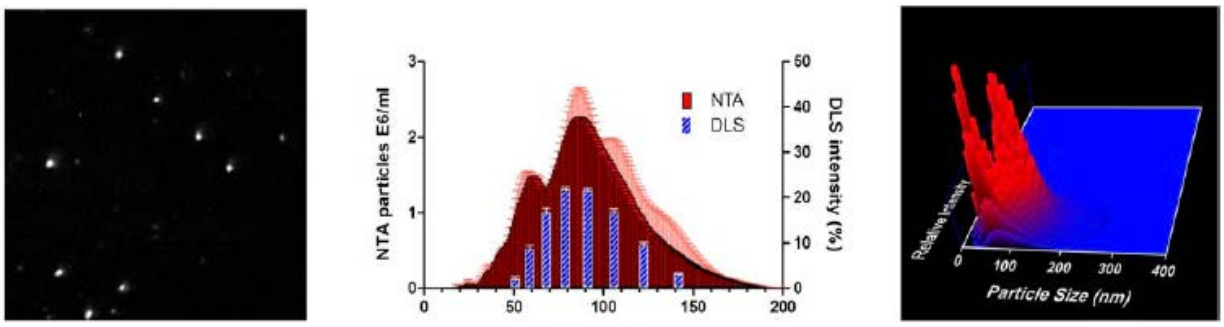

Figure 3(c). Panel on left shows Brownian motion and how it is analysed to count the number and size of the particle. The middle histogram with a graph overlay is a representation of the MV counts generated for a given sample and the panel on the right shows a 3 D plot of particle size. Reproduced from reference.[89]

The interrogation of TRPS and NTA was considered the next relevant step as emerging tools to investigate circulating MV. Each of these approaches has its advantages and disadvantages – these are discussed in the review which follows.

PAPER 2: Enjeti AK, Ariyarajah A, Warwick E, Seldon M, Lincz LF Challenges in Analysis of Circulating Extracellular Vesicles in Human Plasma Using Nanotracking and Tunable Resistive Pulse Sensing. *J Nanomed Nanotechnol*. 2017; 8: 468.

Key learning and reflections

Biological fluids such as plasma are complex and heterogeneous. Repeated measures using several settings or analytical parameters may be required to capture heterogeneity when using novel methods such as TRPS or NTA.

Note : the publications are embedded as pdf and retain their original page numbers as in the manuscript.

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Research Article Open Access

Challenges in Analysis of Circulating Extracellular Vesicles in Human Plasma Using Nanotracking and Tunable Resistive Pulse Sensing

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Abstract

Aim: Extracellular vesicles (EV) are secreted from both healthy and diseased tissues and are detectable in most body fluids, where their measurement can be of prognostic/diagnostic value. We aimed to evaluate pre-analytical and analytical variables in the measurement of EV in human plasma using nanotracking analysis (NTA) and tunable resistive pulse sensing (TRPS).

Method: Commercial beads (200 nm and 400 nm in diameter) and human plasma from volunteer donors were used in this study. A total of 36 bead and 175 plasma measurements were undertaken by NTA and TRPS. The pre- and analytical conditions tested on plasma samples were: fresh, stored for 1 day or 1 week at 4°C or -80°C. The other variables included testing of neat or pelleted EVs and choice of diluent.

Results: The 200 nm and 400 nm beads, when tested alone or combined showed coefficient of variations (CV) of <10% at all dilutions. The CVs for triplicate results of plasma samples varied between 3-43%, with samples frozen for 1 week showing the least CV spread (5-15%). The EV counts from pellets or supernatants obtained after centrifugation of plasma at 21000*g* for 1 h, were different to the neat samples. Although both the supernatant and pellet fractions contained EVs of approximately 100 nm in size, only the pellet contained larger vesicles of 300 nm, and only the supernatant contained vesicles <100 nm.. Different EV counts were obtained for the same plasma aliquot using NTA and TRPS.

Conclusions: Measurements by NTA can be performed on fresh and/or frozen samples provided similar storage and centrifugation conditions are used. The CVs for plasma samples are high indicating the need for standardized conditions. TRPS requires at least two different nanopore filters for measurements and plasma as diluent in lower chamber for optimal results. NTA and TRPS measure EVs by different approaches and the knowledge of the size range for measurement by each method is critical.

Keywords: Extracellular vesicles; Microvesicles; Plasma; Nanotracking analysis; Tunable resistive pulse sensing

Introduction

A variety of extracellular vesicles (EVs), which are membrane bound vesicles released by cellular elements, play an important role in both normal and pathophysiology of the human body. Particles of any size in circulating plasma are now referred to collectively as extracellular vesicles [1]. The term 'microparticles' (MP), used previously in literature to denote platelet derived vesicles, is generally now re-designated as microvesicles (MV) and refers to vesicles in the size range of approximately 100 nm-1 μm. Exosomes on the other hand specifically refer to vesicles released by exocytosis and usually in the size range of 30-100 nm [2].

The existence of cell derived membrane bound particles in human circulation has been described for over two decades [3]. The clinical and pathophysiological significance of these particles has been unravelled in the last few years. These vesicles are usually derived from human blood cells such as platelets, white cells and red blood cells but more recently, EV derived from cancer cells and other tissues have also been reported [4]. The measurement of circulating EV have been described to be of prognostic and diagnostic importance for several diseases particularly in haemostatic/coagulation disorders, cancers and certain inflammatory disorders [5]. Circulating EV can be detected by analysis of human plasma with methods such as flow cytometry, enzyme linked immuno-absorption, as well as functional studies [6,7].

Standardized flow cytometry can determine the number of vesicles as well as antigenic specificity which can help determine the cell of origin. An example of flow cytometric approach includes using a fluorescently labelled anti-CD41 antibody to identify platelet derived vesicles. The challenge with this technique is the limitation posed by the wavelength of the laser light used, thus particles less 400 nm are difficult to capture by standard flow cytometry [8]. Modified and dedicated flow cytometry may be used to detect vesicles as small as 200 nm [9].

A commonly used functional approach uses a combination of phospholipid based capture on a plate followed by functional thrombin production estimation. These assays are not limited by size and hence complement a variety of ways to quantitate EV.

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Evolving technologies to measure EVs now include nanotracking analysis (NTA) and tunable resistive pulse sensing (TRPS). Nanotracking (commercialized as Nanosight NS500 and other versions, Malvern Instruments Limited, Worcestershire, UK) specifically utilizes a combination of microscopic capture of laser light scatter with a charge device coupled camera. A dedicated software program is able to detect dynamic light scattering due to the Brownian motion of individual particles in solution which allows mathematical determination of the size and number of particles [10].

The tunable resistive pulse sensing (TRPS, commercialized as qNano and other versions (Izon Science UK) is a non-optical technique based on the passage of particles through a nano-scale pore. As a vesicle moves through the pore it creates an increase in electrical resistance, which is measured as a blockade height. Mathematical derivations allow the calculation of the vesicle volume from this resistive pulse. Particle by particle detection occurs, enabling size distribution within a sample to be determined. The calculation of particle size by TRPS is dependent on several variables such as pore size, electrolyte used, as well as the stretch, voltage and pressure applied [11].

This article explores the technical challenges as well as advantages of using these two promising techniques for detection and measurement of circulating EV in human plasma.

Methods

General approach to pre-analytical variables for NTA and TRPS

Sample collection: Blood samples were collected in 3.2% sodium citrate from volunteer donors after informed consent. The plasma was immediately separated by centrifugation at 1500*g* for 15 minutes and either processed for immediate EV enumeration or stored at 4°C or -80°C for future experiments.

Processing and analysis: A total of 36 bead and 175 plasma measurements were undertaken with triplicate runs. Commercial beads (200 nm and 400 nm, Izon sciences UK) were analysed in various serial dilutions. The pre-analytical variables for plasma included analysis within 2 h of collection (Fresh), and after storage at 4°C or -80°C for 24 h or 1 week. Other variables tested included measuring neat (no dilution), supernatant and pellets after samples were centrifuged at 21000*g* for 1 hour. The centrifugation conditions were chosen based on commonly employed methods for isolating EV [12]. The statistical analysis was performed using Prism 6 software (Graphpad, USA). Ethics approval for using human plasma samples for this project was obtained prior to commencement of the experimental work (Hunter New England ethics committee approval number 06/12/13/5.05).

NTA for EV in human plasma

Specific pre-analytical variables

Dilutions and linearity for beads and plasma: *Two* bead sizes 200 nm and 400 nm were tested in dilutions ranging from 1:100 to 1:1000 in triplicate measurements. A mixed sample (1:1) of the two bead sizes was also tested in such serial dilutions.

The neat, supernatant or pellet fractions from plasma were tested in triplicate. The nanotracking was performed in both scatter and fluorescent modes in order to distinguish EV from background. Qdot 625 stain (Life Technologies/ThermoFischer Scientific, MA, USA) was diluted 1:100 and incubated with samples at 37°C for 1 h. A negative control (stain in buffer) and unstained plasma was used with every experiment. Events were collected from the samples at the entire range of camera settings from levels 9-16 (based on camera shutter and gain) both for scatter and fluorescent modes with fine tuning of the camera adjusted to best visualize the EVs at the camera setting. The detection threshold was set at 10 and events were captured for 90 s. Results were analysed using the NTA software (Nanosight NTA 2.3 software).

TRPS (qNano) for EV in human plasma

Sample preparation by pre-filtration: A filtration step was required for analysis using TRPS in order to prevent clogging of the nanopore. The 0.8 μm filter and 0.45 μm filters efficiently prepared the sample for analysis on 800 nm and 200 nm nanopores respectively.

Fluid composition in the top and bottom chambers: A combination of Plasma/PBS and plasma/plasma was tested across the nanopore in the qnano instrument to check for optimal results. Dilutions ranging from 1:2 to 1:50 were tested for linearity.

Linearity across dilutions and optimal dilution for plasma samples: We also investigated if plasma dilution was a viable option without impacting on the actual particle count. We used a 200 nm nanopore for the experiments and compared the current (nA) and particle rate with neat, 1:10 and 1:20 plasma samples diluted in PBS.

Stretch and voltage

A fixed blockade height approach and a fixed stretch/voltage approach was compared.

Results: NTA for EV in human plasma

Reproducibility and linearity for beads: A CV of <10% for all dilutions of both 200 nm and 400 nm sized beads was observed and linearity in measurements demonstrated as shown in Figure 1. The bead counts calculated by NTA, upon dilution, varied by two to six-fold compared to expected bead concentrations provided by the manufacturer (data not shown).

Centrifugation and storage conditions for plasma samples: There was no significant difference between EV results for fresh or frozen stored samples under different storage conditions (2-way ANOVA $p>0.05$, Figure 2). A trend for higher EV levels in frozen samples was observed. The CVs for triplicate results of plasma samples varied between 3-43%, with samples frozen for 1 week showing the least CV spread (5-15%).

The EV counts from plasma pellets or supernatants obtained after centrifugation at 21000*g* for 1 h, were reduced compared to the neat samples. However, there was no significant difference between the EV counts in the pellet and supernatant fractions, with particles averaging 5.08 and 7.46 x 10^8/L respectively. To investigate this surprising finding, the vesicle sizes between the fractions were compared by further analysis of the peaks on the nanosight graphical histogram output. The major (first) peak on the NTA histogram was similar for both fractions and measured 107.1 nm and 101.1 m respectively. However, both fractions contained a minor peak, and the median size for this minor (second) EV size peak from supernatant was 94.3 nm, which was significantly smaller compared to the 312.4 nm for the pellet (Figure 3).

Sample dilution: Ideal dilution for plasma samples was determined to be between 1:50 to 1: 100 depending on whether fluorescent or scatter events were captured. In lower dilutions, it was easier to detect fluorescent vesicles/particles, as there tended to be fewer events than in the scatter detection mode. When the number of tracks captured was between 40-200, the CVs were tighter. Dilutions in the range of

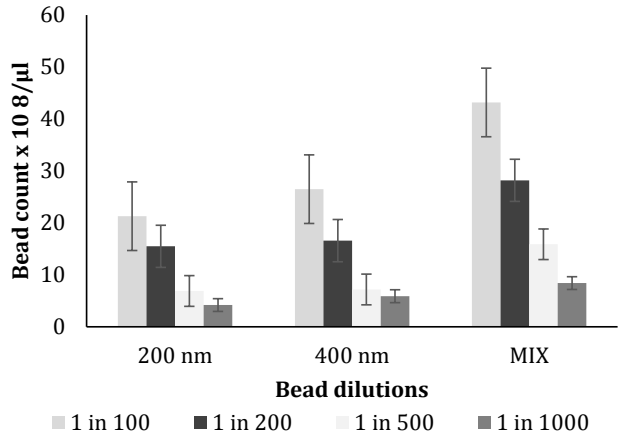

Figure 1: Bead counts for 200 and 400 nm beads in various dilutions with mean and error bars (±2 SD).

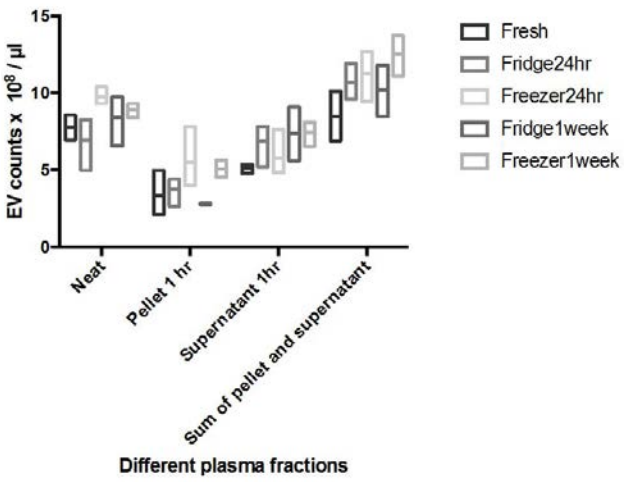

Figure 2: Comparison of EV counts obtained from neat, pellet and supernatant fractions obtained after centrifugation at 21000*g* for 1 h.

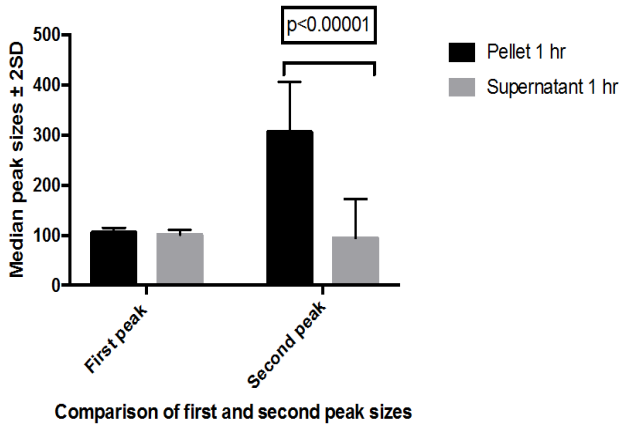

Figure 3: Comparison of largest and second largest peak sizes in EV from pellet and supernatant after centrifugation at 21000*g* for 1 h (n=6), frozen samples.

1:1000 or 1:10000 were required for samples where the number of tracks exceeded 200. The instrument defaults to water's viscosity (0.97 cP at 23.3°C) in its custom settings and may need to be adjusted to the viscosity of the sample being run.

Complex samples-plasma at different camera levels: As the camera level and filter varied from fluorescent to light scatter, adjustments on the instrument were required to be made before the events were captured. Most bead or plasma events appeared to be best detected at camera levels of 13-15, with background scatter increasing with camera level (Figure 4). Often camera levels at 16 (highest possible level) impeded any detection of vesicles or particles due to significant background scatter. With each camera level fine tuning of the focus was also critical to achieving maximum capture.

Results

Technical aspects of TRPS (qNano) for EV in human plasma

Electrolyte and conductivity: The electrolytes used in the experiments directly influence the conductivity and therefore the rate of particle movement across the nanopore. For example, the particle rate will be higher flowing from a solution that is more charged to a solution with less charge than if both chambers have the same solution. Table 1 shows the comparison of plasma in both the chambers as compared to plasma in the top and PBS in the bottom chamber.

Linearity across dilutions and optimal dilution for plasma samples: The particle rate was high when plasma was used undiluted in the upper chamber (Table 2), leading to repeated pore clogging and interruptions to the experiments. This problem was ameliorated when the plasma was diluted, which also increased the current across the nanopore. Longer acquisition times and different settings were required for diluted samples and up to 90 s was used to capture more events; viscosity settings needed to be adjusted to the medium of suspension (e.g. changed to PBS=1.03. cP).

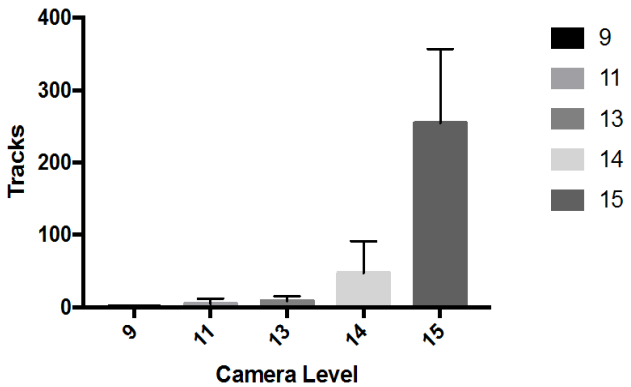

Figure 4: Tracks captured in fluorescent mode for plasma EV at various camera levels (in triplicate, with error bars showing SD).

Combination of fluids in upper and lower chambers	PBS/Plasma (bottom/top)	Plasma/Plasma (bottom/top)
Avg Current (nA)	102.3 ± 3.54	109.58 ± 2.1
RMS noise (pA)	11.93 ± 0.51	11.8 ± 0.4
Particle Rate (particles/min)	3257.63 ± 1589.11	2395.9 ± 1433.67

Voltage=0.6V Stretch=45.67 Pressure=7.

Table 1: Electrolyte and conductivity across chambers (n=3).

Using a combination of pre-filtration for polydisperse plasma samples: A pre-filtration step with a 0.8 μm syringe filter for the 800 nanopore and a 0.45 μm syringe filter for the 200 nanopore was employed to detect particles of various size ranges based on the upper limit of detection for the respective nanopore. The stretch (42.8 nm) and voltage 0.32 V was kept constant across all measurements as per manufacturer's instructions and published literature [11,13].

Nanotracking and TRPS - Do they measure the same vesicles?

Nanotracking and TRPS employ different technologies and hence measurements made with either instrument may reflect different populations of particles. The table below demonstrates that the respective optical and non-optical measurements by nanotracking and TRPS can vary on the same sample and pre-analytical preparation combination (centrifugation, pellet vs. supernatant and filtration) as shown in Table 3.

Discussion

Emerging techniques of NTA and TRPS have been increasingly used in detection and quantitation of EV of biological origin [14]. Although there is a significant body of literature looking at beads and liposomes potentially simulating body fluids there is a dearth of published literature estimating EVs in human plasma samples [13]. We investigated the pre-analytical as well as analytical variables for human plasma measurements by NTA and TRPS, hypothesizing that this would be more complex than bead or liposome measurements. The key findings indicate that standardized pre-analytical processing such as storage and centrifugation as well as knowledge of particle size range is critical for measurement of EVs in human plasma. Some of the specific findings are discussed in the sections below.

Pre-analytical variables for measuring EV in plasma using NTA or TRPS

Pre-analytical variables for both techniques can significantly impact on the results. Sampling of blood for functional and numerical assessment of circulating cell-derived vesicles has been shown to be best achieved by citrated or heparinised samples [15]. Though EDTA may be sufficient as an anticoagulant and preferable for RNA based experiments, it is likely that any concomitant EV testing involving coagulation, thrombin generation or functional experiments cannot be undertaken on the same sample.

	PBS/Plasma neat (bottom/top)	PBS/Plasma 1:10 (bottom/top)	PBS/Plasma 1:20 (bottom/top)
Avg. Current(nA)	97.26	118.27	122.65
RMS noise (pA)	10.46	7.42	7.74
Particle Rate (particles/min)	360.2	190.8	145.1
CV (%) for Avg. current	30.3%	4.5%	1.0%

Table 2: Plasma samples showing differences in CV with various dilutions, n=3.

Sample	TRPS particles × 10^8	NTA particles × 10^8
Neat	23	4.4
Pellet 1 h	14	8.1
Supernatant 1 h	17	5.78
Pellet 10 mim	11	2.87
Supernatant 10 min	14	7.38

P<0.0001 for the two sets of results in all rows.
All plasma samples subjected to 800 μm pre-filter.

Table 3: Comparison of NTA and TRPS, on pre-filtered plasma, for varying pre-analytical conditions.

A neat (i.e. not centrifuged) sample is theoretically sufficient for measuring all EVs in the plasma sample, however, we observed that for analysis by NTA and TRPS, diluted plasma samples provided more optimal measurement conditions. The quantitation of a polydisperse mix of vesicles within a given sample is challenging given the finite size range within which each technique operates. Bench top centrifuges achieve a force of up to 21000*g* which is enough to achieve pelleting of a significant proportion of larger EV (mostly microvesicles and some exosomes), however small <100 nm and/or less dense EV are not pelleted efficiently. The examination of MV, the fraction of EV that lie between 100 nm-1000 nm, as seen by the size of the second peak by NTA in our experiments, is potentially facilitated by centrifugation at 21000*g* for 1 hour to pellet these vesicles. Centrifugal forces greater than 100000*g* will achieve isolation of the exosome and small vesicle pellet (vesicles in the size of 100 nm or smaller) [16].

NTA

We evaluated the pre-analytical variables of storage and centrifugation. Lower counts of vesicles in the pellet compared to the supernatant fraction or neat plasma may reflect the fact that a centrifugation speed of 21000*g* results in isolation of all large (which would generally fit the description of endovascular or platelet derived MV) and some smaller, perhaps denser EVs, however, smaller vesicles, including exosomes, and plasma lipoproteins are not likely to be pelleted. Commercial beads can be used to simulate conditions for microvesicle estimation, however plasma samples are more challenging to analyse due to heterogeneity in EV size. EV measurement on Nanosight can be performed on fresh and/or frozen samples whilst ensuring that the pre-analytical variables are consistent. The CVs for plasma samples are high (up to 43%) indicating the need to run them in replicates. This will help reduce the high inter-run variability and average value could be considered as close to the true measurement. Solvent parameters need to be adjusted for NTA to ensure the conditions for temperature and viscosity are maintained in all experiments.

Freeze-thaw may interfere with vesicle integrity, as shown by higher EV counts in the frozen preparations. The size range detected in the pellet fractions suggest that both small and larger EV in the nm range is pelleted. The presence of EVs in the supernatant suggests some vesicles or plasma proteins cannot be pelleted and will be detectable on nanotracking. It is also likely that the exosome fraction is incompletely pelleted by 21000*g* for 1 h as shown by the presence of <100 nm EV in the supernatant fraction. It is therefore important to treat all samples in a similar fashion with respect to anticoagulant, centrifugation and storage conditions in order to minimize pre-analytic variability.

TRPS

One of the challenges using TRPS for plasma microvesicle measurement is the limitation imposed by the size of nanopores and the corresponding particle size range that can be measured. Nanopores (NP) are conical holes formed in flexible polyurethane membranes which are stretched to optimise the nanopore size on the qnano instrument according to the particles being measured. For example the 200 nm nanopore will measure a size range between 85-500 nm whereas a 800 nanopore will measure a size range between 385-2050 nm [13]. Analysis of an exosome population will require a nanopore of 70 nm (analysis range size 40-255 nm), 100 nm (analysis size range=80 to 255 nm) or 150 nm (analysis size range=70 to 420 nm). Usually, the manufacturer quoted particle range cannot be detected at single stretch of the nanopore [17].

Another technical challenge with using nanopores for analysis of heterogeneous samples such as plasma is that the smaller nanopores tend to get clogged with large particles. A filtration step was required for analysis using TRPS to detect vesicles of a certain size. The 0.8 μm and 0.45 μm syringe filters efficiently prepared samples for runs on the 800 nm and 200 nm nanopores without causing clogging during analysis. The particle rate was high when plasma alone was used undiluted in the upper chamber with the average current that flows across the pore being lower. This can also lead to repeated pore clogging which could interrupt the experiments. High dilutions resulted in very low particle rate and incorrect or inaccurate concentration estimates. Therefore, based on our observations a 1:10 or 1:20 dilution appears optimal. Stretch and voltage was fixed for our TRPS experiments based on trial runs and published literature from urinary vesicles [14].

Variability in measurements and techniques

The EV concentration and detectable size range can vary between techniques and instruments used. Variability in vesicle concentrations is primarily caused by differences between the minimum detectable vesicle sizes. The minimum detectable vesicle sizes reported in literature are 70-90 nm for NTA, 70-100 nm for TRPS, 150-190 nm for dedicated flow cytometry, and 270-600 nm for conventional flow cytometry. Transmission electron microscopy (TEM) has the potential to detect vesicles which are smaller in size, however only after they are adhered on to a surface. Dedicated flow cytometry is more accurate in determining the size of reference beads, but may have less accuracy when measuring biological vesicles, owing to heterogeneity of the refractive index of vesicles from body fluids or cells [18]. The main role of NTA and TRPS is to facilitate the measurement of EVs in the size range of 100 nm-300 nm which are usually not measured using standard techniques such as flow cytometry [1,14]. It may be critical to accurately measure vesicles in experiments where a correlation between particle count and function of EV is being ascertained [19]. One recommendation is to ensure that any reported concentration is accompanied by the minimum detectable vesicle size. Alternatively, mathematical modelling using a power-law fit can also be performed based on detectable minimum size [1].

Other authors have trialled spiking biological fluids with polystyrene beads of known size and concentration to improve EV measurement accuracy [19,20]. For NTA, a similar approach using silica beads would be less useful as the scatter mode does not discriminate between particles of similar size. We observed in our experiments that calculated bead counts show 2 to 6-fold variance from expected counts, particularly in diluted samples where the concentration of beads may not be optimal for the method employed. In fact, most manufacturers of calibration beads do not recommend dilutions given the errors that can be introduced in the process [17]. Also, for either mode, spiking samples with large (>500 nm) silica beads could lead to blockages of nanopores on TRPS and over scattering of the EVs as well as skew the characterization by NTA [18,21]. Applying correction factors for calibration beads mixed into biological samples is easier to apply in homogenous samples but more challenging in a complex sample such as plasma. Assessing subpopulations of varying sizes may be challenging by either method unless a complex mix of calibration beads of varying sizes can be independently applied to accurately quantitate. Such a process needs extensive validation before it can be applied to biological samples. However, measuring one particular narrow size range, by either technique, is possible based on the analytical limits of the process applied, e.g. pre-filtration and selecting nanopore size for TRPS, pre-filtration and focusing on one peak size for NTA.

Concluding notes on applications of nanotracking and TRPS for assessment of circulating MV or EV in plasma

The focus in vascular biology and haemostasis research is usually to evaluate the contribution of vesicles derived from platelets, as well as white, red and endothelial cells to the haemostatic process. Endothelial integrity and function can also be evaluated by measuring endothelial derived EV in circulation. The size range for studying endovascular vesicles in circulation is quite wide -ranging from a few nanometers up to 1 μm [22]. There are several reports of cardiovascular risk factors as well as cardiovascular disease being linked to circulating MV or EV - however, the studies have often used widely disparate technologies making it challenging to compare the results [2].

Flow cytometry techniques have been very popular in clinical physiology and vascular biology EV research, however the limitations of flow cytometry are now well recognized [8]. Both nanotracking and TRPS provide alternative techniques which may have potential to overcome some of these drawbacks [23]. Whilst nanotracking has potential for identifying specific EVs labelled with Qdots, TRPS has the potential of being able to mark EVs with aptamers. TRPS also has potential for particle by particle measurement of surface charge (known as zeta-potential) which may provide a discrimination between particles of varied surface charge and surrogate evidence for different cells of origin for the vesicles [11,14].

The potential benefits for sizing biological nanoparticles via resistive pulse sensing with a tunable nanopore has been recently demonstrated in experiments using adenovirus for chemotherapy delivery. The relation of size to physiological or pathophysiological role of the EVs is not clear; though it is possible that certain sized biological vesicles have greater capacity for cargo (either RNA or protein) transfer [12,23]. It is important to understand that biological fluids such as plasma are complex and heterogeneous. Repeated measures using several settings or analytical parameters may be required to capture the heterogeneity in a single sample and may still be unable to accurately measure vesicles in all size ranges. Understanding the advantages as well as limitations of newer approaches such as NTA and/or TRPS will enable focused, more accurate and comprehensive assessment in a specific size range of EV in circulation.

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Appendix PAPER 3:

Enjeti AK, Lincz L, Seldon M. BioMaleimide as a Generic Stain for Detection and Quantitation of Microparticles. *International journal of Laboratory Haematology*. 2007; Jul 2(3):196-199.

Key Learning points

Generic MV stains such as annexin V have limitations and other approaches such as using Biomalemide may be considered for generic detection of MV. Specific markers are still required to determine the cell of origin for MV.

Note : the publications are embedded as pdf and retain their original page numbers as in the manuscript.

Bio-maleimide as a generic stain for detection and quantitation of microparticles

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Keywords
Microparticles, thrombosis, maleimide, quantitation, analytical variables

SUMMARY

Microparticles (MP) are small fragments of cytoplasm shed from a cell surface and their role in the pathophysiology of disease is being extensively investigated. A novel staining technique for quantifying total MP in peripheral blood was evaluated in this study. Evaluation of Bodipy-maleimide (or bio-maleimide) as a stain for quantifying total MP in peripheral blood by flow cytometry. Samples were obtained from 10 healthy donors after informed consent. Plasma was prepared by sequential centrifugation at 1500 *g* followed by 13 000 *g* and stained with Annexin V and bio-maleimide. Enumeration beads were added after 15 min of incubation with the stain and samples analyzed on a FACS Canto flow cytometer. Detection and quantification of MP by bio-maleimide staining was comparable with that by Annexin V. The total mean MP level with bio-maleimide staining was 34 ± 19.7/μl (range of 11.6–68.1/μl) and with Annexin V staining it was 38.9 ± 29.8/μl (range of 10.6 to 112.9/μl). There was no significant difference using a paired *t*-test and methods were comparable using a Bland–Altman plot. Bio-maleimide is a useful and inexpensive stain to measure total MP levels in peripheral blood by flow cytometry. This technique could be employed to study thrombotic risks in a variety of disease states.

INTRODUCTION

Microparticles (MP) are small fragments of cytoplasm which are shed from a cell surface (Freyssinet, 2003). These are released from activated or dead (apoptotic) cells. They have been until recently considered inert cell debris. It now appears that they have considerable pathophysiologic potential. Microparticles have been defined as membrane bound particles ranging from 0.1 to 1 μm in size expressing surface markers of it's cell of origin (Working group on vascular biology, ISTH 2005).

The production of MP is said to occur by vesiculation or blebbing of the cell membrane and is thought to reflect a balance between cell stimulation, proliferation and death (Freyssinet, 2003). In recent years, measuring MP has increasingly received attention both as a diagnostic aid and investigative tool. Different methods employed include flow cytometry, solid phase capture and ELISA (Horstman *et al.*, 2004).

Levels of MP are reported to be higher in patients with venous as well as arterial thrombosis (Chirinos *et al.*, 2005; Namba *et al.*, 2007) when compared with healthy subjects. Abnormal amplification of MP production may lead to a pathological state for example, excessive MP from platelets may contribute to thrombosis. In fact, a recent review has emphasized MPs as key players in thrombosis by including them as a part of triad for thrombosis (Polgar, Matuskova & Wagner, 2005). Thus, quantifying MPs may be important in assessing the thrombogenic profile of a person.

The International Society for Thrombosis and Haemostasis invited a forum in 2004 to report on MP detection and quantitation (Jy *et al.*, 2004). A number of different detection methods were discussed in the forum. This study was undertaken to evaluate a novel stain bodipy-maleimide (hence forth referred to as bio-maleimide) for enumeration of total MP in peripheral blood samples and compare it with traditional methods for detection of MP. This stain has been shown to detect MP of platelet origin but has not been evaluated for obtaining a total MP count (Dale, Remenyi & Friese, 2005).

METHODS

Consent and ethics

This study was carried out in compliance with the protocol and in accordance with the National Statement on Ethical Conduct in Research Involving Humans. The study was reviewed and approved by the Hunter Area Ethics committee. Written informed consent was obtained from each participant prior to registration on the study. Samples were obtained from participants invited to be part of the study at the Newcastle Mater hospital after receiving informed consent.

Preparation of samples

Blood samples were drawn into buffered citrate after discarding the first 3 ml and transported to the laboratory. Platelet free plasma (PFP) was prepared by a two-step centrifugation procedure: 1500 *g* for 15 min at 20 °C to make cell free plasma and then once at 13 000 *g* for 2 min at 20 °C to make PFP. These samples were fresh or aliquots of MP (500 μl) stored at −80 °C for further experiments.

The following antibodies were used

- Marker of activation/phosphotidylserine expression: Annexin V conjugated to FITC (BD PharMingen, San Diego, CA, USA)
- Gating beads: 0.5 μm and 1.0 μm (Flouresbrite Microspheres; Polysciences Inc., Warrington, PA, USA)
- Counting beads 10 μm beads(Flow–count Flourospheres; Beckman Coulter, Fullerton, CA, USA)
- Bio-maleimide (Bodipy FL *N*-(2-aminoethyl) maleimide, no. B-10250): Molecular probes; Invitrogen, Carlsbad, CA, USA.

Negative controls were used in Annexin V experiments.

Flow cytometric detection of microparticles

All reagents and solutions used were sterile and filtered (0.2 μm filter). The 50 μl of MP suspensions were incubated with 5 μl of Annexin V in a final volume of 100 μl phosphate buffered saline containing 2.5 mmol/l of $CaCl_2$ for 15 min in the dark. The sample was diluted to 400 μl after the incubation process by adding filtered PBS. Enumeration beads (known number of 10 μm beads in a volume of 100 μl) were also added after 15 min of incubation time. Sizing beads were used to set initial size gate.

Bio-maleimide was dissolved at 5 mM in dimethylsulfoxide and 20 μl aliquots were stored at −80 °C. Bio-maleimide was diluted 1 : 100 in buffered saline before use and 5 μl was added to 100 μl of sample. It was used in a final concentration of 2.5 μM and 5 μl was incubated with 50 μl of MP suspension in a final volume of 100 μl phosphate buffered saline containing 2.5 mmol/l of $CaCl_2$ for 15 min in the dark. Enumeration and sizing beads were added as described above.

Data were acquired and analyzed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) with appropriate software. MPs were simulated using commercially available beads of the appropriate size. The MP gate, based on a particle size of <1.0 μm, was used for identification of MP. Events in the MP gate were assessed for labeling with antibody positive events to distinguish true events from electronic noise. MP quantitation was done using a modified Combes method (Kim *et al.*, 2002). The MP were enumerated by using the formula:

$$(10/9) \times (\text{microparticle count/bead count}) \times (\text{bead concentration}/\mu l) = \text{MP count}/\mu l$$

Statistical analysis was carried using the *t*-test for paired data. The differences were considered significant if $P < 0.05$.

RESULTS

Ten healthy participants were recruited. The average age of the participants was 52.1 years and the male to female ratio was 3 : 2. The participant characteristics are given in Table 1.

The mean (±SD) MP counts obtained on samples using Annexin V staining was 34.0 ± 19.7 MP/μl compared with 38.9 ± 29.8 MP/μl obtained with bio-maleimide. There was no significant difference between mean MP counts using Annexin V or bio-maleimide staining (paired *t*-test, $P = 0.27$). The results for both the stains are shown in Table 2. The two methods were found comparable by Bland–Altman statistics (as shown in Figure 1).

The cost of using the novel stain was Australian $4.00 for a 100 tests in comparison to Australian $40.00 for the same number of tests using Annexin V. Aliquots of the bio-maleimide stain can be frozen at −80 °C for long-term use (data not shown).

Table 1. Baseline characteristics of participants in this study

Feature	Mean	Range
Age	52.1 years	40–65 years
Hemoglobin	144.8 g/l	130–158 g/l
Albumin	40.1 g/l	37.8–42.4 g/l
Platelets	265.5×10^9	$157–468 \times 10^9$

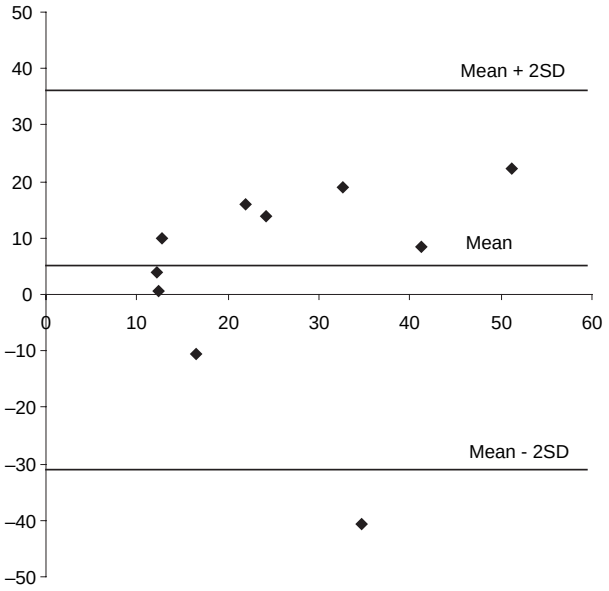

Figure 1. Difference of values obtained by the two stains (Annexin V and bio-maleimide) on the *x*-axis *vs.* their average on the *y*-axis (Bland–Altman plot). The mean represents the mean of the difference and the SD represents the standard deviation of the differences.

DISCUSSION

Bio-maleimide has evoked interest because of its property to attach to biological membranes via cysteine residues and thiol groups in proteins and its fluorescent nature enables its use in flow cytometry (http://www.probes.com Accessed January 2007). It has previously been shown to detect platelet MP produced *in vitro* from platelets; however, has not been used to demonstrate circulating MP in human plasma (Dale, Remenyi & Friese, 2005). Table 3 compares the properties of bio-maleimide and Annexin V.

Several international groups have been working towards better techniques to detect and analyze MP

Table 2. Comparison of microparticles (MP) counts obtained by using Annexin V and bio-maleimide

Stain used in flow cytometry for detection of MP	Mean MP count MP/μl of plasma ± SD ($n = 10$)	Range of MP count/μl of plasma ($n = 10$)
Annexin V	34.0 ± 19.7	11.6–68.1
Bio-maleimide	38.9 ± 29.8	10.6–112.9
P-value (paired *t*-test)	0.27	–

Table 3. Comparison of Annexin V and bio-maleimide as stains for detection and quantitation of microparticles

Properties/stain	Annexin V	Bio-maleimide
Chemical nature	35–36 kDa Ca^{2+}dependent phospholipids binding protein conjugated to FITC	BODIPY-fluorophores bound to maleimide which has affinity for thiol groups
Staining affinity	Exposed phosphotidyl-serine on cell membrane	Cysteine residues and thiol groups in cell membranes
Emission spectrum	500–540 nm	500–540 nm
Final concentration	5 μl of staining solution/needs $CaCl_2$ during staining	2.5 μM/reconstituted in DMS0
Cost/100 tests	A $40.00	A $4.00
Storage	At 2–8 °C till expiry (approximately 2 years)	At −80 °C in aliquots for several years

(Kim *et al.*, 2002; Jy *et al.*, 2004). There is a need to standardize the preanalytical/analytical variables as well develop new more cost effective methods for detection and quantitation of MP. The use of bio-maleimide is akin to using a screening test to look for markers of disease (e.g. raised total MP count) which if abnormally raised can be further evaluated by specific antibodies based on the clinical history (e.g. measuring platelet and endothelial MP in a person with history of stroke).

The results of this study show that it can be used instead of the more expensive monoclonal antibodies to measure total MP in peripheral blood. The results obtained with the use of bio-maleimide are comparable with those obtained with Annexin V staining, the latter being considered the standard generic stain for MP. The inability to detect the cell of origin would a drawback of using bio-maleimide. However, it would be useful for screening for elevated levels of MP in human plasma samples, which could then be analyzed further using specific antibodies. The low cost and easy long-term storage would make it an economical screening technique for detection of MP.

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Understanding of the methods and technologies for measuring MV/EV provided the basis for the next chapter which discusses the role of MV in health.

CHAPTER 4

MICROVESICLES IN HEALTH

4.1 Microvesicles in healthy 'normal' subjects

There is limited information about the variability of MV in normal subjects despite the extensive literature in pathological states. A couple of small studies have described higher levels of MV in females.[90] In a more recent study, the diurnal variation of MV in a small sample of six healthy donors was observed.[84] Another study, which also used flow cytometry for quantitation, showed that there was a postprandial rise in circulating MV compared to a fasting state.[91] There is also a suggestion that increased levels of MV after exercise may have a potential cardio-protective role in animal models.[92]

A comprehensive profiling of MV in normal healthy subjects is critical for the understanding of their physiological role before pathophysiological assumptions can be evaluated. The association of MV with age as well as the impact of factors such as lipids, hormones, smoking and use of drugs such as aspirin on circulating MV levels has not been extensively investigated. Baseline coagulation assays in clinical use such as PT (prothrombin time), PTT (activated partial thromboplastin time) and TT (thrombin time) measure the contribution of common plasma proteins to the process of coagulation measured usually by the end point of fibrin clot formation. Modification of certain tests can be adapted for measuring MV such as the XaCT test which measures the Xa formation in phospholipid poor plasma and therefore indirectly measures contribution of MV derived phospholipid for Xa generation. More specialised tests are required to measure the level, cell of origin (flow cytometry, NTA and TRPS) and function (ELISA and thrombin generation) of MV. The details of these more specialised tests are discussed in detail in chapter 3.

A literature review revealed that there was lack of comprehensive data from normal health subjects across a wide age. We therefore felt it was critical to evaluate the MV distribution in normal healthy subjects. It is debatable as to what the normal range of MV in circulation should be given the range of known and unknown variables that may potentially influence its level. Not only is the knowledge of method variability important, but also understanding biological variability becomes critical when samples from disease groups are evaluated. Before comparisons of normal to abnormal samples are made, this part of the project was undertaken to understand the variability – both methodological and biological - in a cohort of 'normal' subjects. Whilst the previous chapter focussed on inter-method variation, the following chapter addresses various aspects of biological variation.

Aim 2:

To undertake a comprehensive analysis of variables that affect circulating MV in normal healthy subjects, specifically focussing on age, gender, lipid /hormone profile and smoking status.

PAPER 3: Enjeti AK, Ariyarajah A, D'Crus A, Seldon M, Lincz LF. Circulating microvesicle number, function and small RNA content vary with age, gender, smoking status, lipid and hormone profiles. *Thromb Res.* 2017;156:65-72.

Key learning and reflections

High baseline variation in MV numbers and function was observed in a cohort of normal subjects, reflective of individual biological variation.

Key independent predictors of MV parameters include age, gender, smoking status and platelet counts.

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Full Length Article

Circulating microvesicle number, function and small RNA content vary with age, gender, smoking status, lipid and hormone profiles

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ABSTRACT

Background: Characterization of circulating microvesicles (MV) in healthy subjects in relation to various biological factors is not well studied.
Objectives: We evaluated the influence of age, gender, smoking status, lipid and hormone profiles on circulating MV in healthy subjects.
Methods: Platelet free plasma from 143 volunteer blood donors (males = 80, females = 63) was evaluated by standardized flow cytometry for MV expressing CD41 (platelet-derived), CD105 (endothelial-derived), CD235 (red cell-derived), TF (tissue factor) and phosphatidylserine (PS) MV. Procoagulant function was measured by the Xa based assay (XaCT) and endogenous thrombin potential (ETP) using thrombin generation assay.
Results: Those ≤29 years and ≥60 years had higher levels of MV subsets (CD41, CD235, TF and PS) compared to those aged 30–59 years. The median CD41, CD105, CD235, TF and PS expressing MV by flow cytometry were similar or lower in females, whilst procoagulant activity by the XaCT assay was higher ($p = 0.002$). In smokers ($n = 21$), certain MV subsets (CD41, TF and PS) and functional activity (ETP) was lower ($p < 0.05$). Regression analysis showed that MV parameters of CD41, CD105, TF and ETP could be predicted independently by age, whilst smoking predicted for CD105, CD235, TF, PS and ETP. Certain MV parameters also correlated with BMI, lipid and hormone levels. The small RNA and miRNA levels did not differ by age group, smoking status or gender.
Conclusions: It is important to recognize that differences may arise depending on age, gender, BMI, lipid, hormone levels and smoking status in apparently healthy subjects when evaluating MV for pathogenic potential.

1. Introduction

Circulating microvesicles are known to influence patho-physiological processes in several different ways. Their cell derived membranes carry remnants of proteins that can interact with ligands on other cellular surfaces, allowing their packaged cargos of intracellular proteins and nucleic acids to be transferred to awaiting cells. They are generally procoagulant, able to initiate coagulation through the expression of tissue factor, or simply provide a surface for the assembly and activation of coagulation protein complexes [1,2]. When describing small submicron circulating particles, the terms 'microparticles' (designated as MP) or 'microvesicles' (MV) have both been used interchangeably. The term 'exosomes' is usually reserved specifically for vesicles <50 nm in size that are derived from multivesicular endosomes [3]. We have employed the term 'MV' to designate any cell derived anucleate particle in circulation in the range of 1000 nm or less in size.

Accurate enumeration and characterization of MV ex vivo remains a challenge, with results largely influenced by the different methods of blood collection, transport, processing and storage currently being used in various laboratories. MV in circulation have been widely reported to be associated with various disorders causing increased risk of thrombosis [4,5]. Although several studies have evaluated the pre-analytical variables that can affect MV measurements, very few have comprehensively addressed the normal variability in healthy subjects with respect to age, gender, body mass index (BMI), lipid and hormone profiles, or even smoking status [6,7]. The aim of this study was to conduct a detailed evaluation of MV subsets and procoagulant function in healthy individuals and to determine demographic, clinical and biochemical parameters that influence MV levels, function and small RNA content.

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2. Methods

Blood samples were collected from 155 healthy volunteer blood donors after receiving informed consent. Subjects with any significant history of clinical cardiovascular disease, cerebrovascular disease, thromboembolic disease (deep vein thrombosis or pulmonary embolus), dyslipidemia (LDL cholesterol >190 mg/dl); current use of lipid-lowering medications, uncontrolled hypertension (systolic BP >150 and/or diastolic BP >95), chronic diseases including any cancer (other than limited stage skin cancers), renal failure, cirrhosis, diabetes mellitus, endocrinopathies and any known infections or at high risk for infections were excluded (n = 12).

2.1. Sample preparation

Peripheral blood was collected in 0.109 M tri-sodium citrate. Platelet-free plasma was prepared by double centrifugation of whole blood for 15 min at 2500 ×*g*. All samples were processed within 2 h of collection, and aliquots were stored at −80 °C for further batched analysis. Samples were then thawed at 37 °C, 15 min before testing.

2.2. Flow cytometry

Combinations of platelet marker (CD41a-PE; Clone HIP8, BD Biosciences, CA, USA), red cell marker (CD235a-APC; Clone GA-R2, BD Biosciences, CA, USA), endothelial marker (CD105-PE; Clone IG2, Beckman Coulter, Marseille Cedex, France), and tissue factor (TF-FITC; Clone VD8, American diagnostics Inc., CT, USA) or appropriate isotype controls was mixed with a 10 μl aliquot of platelet-free plasma diluted in 100 μl of PBS and incubated (in the dark) at room temperature for 15 min. For experiments with annexin V-APC (eBioscience, CA, USA) for detecting PS the incubation was done in a total of 50 μl of calcium rich binding buffer. All the samples were diluted to 500 μl with filtered PBS or of calcium rich buffer as appropriate. A predetermined number of 10 μm enumeration beads (CountBright beads, Molecular Probes, Life Technologies, Oregon, USA) was added prior to analysis and gating strategy as well as calculations shown in the appendix [8]. We had previously participated in the 'ISTH workshop for standardization of flow cytometry for Microparticles' [9], and the flow cytometer (BD FACS Canto, BD, New Jersey, USA) gating was set according to these guidelines using Megamix beads (Biocytex, Marseille, France). The analysis was undertaken using FACSDiva software.

2.3. Functional coagulation based studies

The factor Xa activation test (XaCT) was performed using the commercially available XaCT test kit (Haematex, Australia) [10]. This is a clot-based assay which detects procoagulant activity of MV, based on the ability of vesicles expressing phosphotidylserine to generate Xa (expressed in ng/ml). It was performed in duplicate and the results were recorded on an automated coagulation analyzer (BCS, Siemens Healthcare, Erlangen, Germany).

2.4. Calibrated Automated Thrombogram measurements

Thrombin generation experiments were carried out on the Calibrated Automated Thrombogram (CAT) and the data was analyzed on the Thrombinoscope software version 3.0029 (Thrombinoscope, Stago Group, Maastricht, The Netherlands). In summary, 80 μl of platelet free plasma was incubated with 20 μl calibrator or 20 μl of specific MP-reagent (Thrombinoscope, Maastricht, The Netherlands). The CAT automatically dispenses the prepared fluorescent substrate and buffer. Thrombin generation was detected, measured and analyzed by the instrument's software to generate the endogenous thrombin potential (ETP) reading.

Table 1
The demographic and baseline full blood count profile of the 108 subjects.

	Mean	Std. deviation	Median	Interquartile range
Age (years)	40.4	15.8	41	25.25–53
BMI (kg/m^2)	26.8	4.4	26.4	24.3–28.4
Hb (g/L)	129.5	11.2	131	121–138
WCC (10^9/L)	6.1	1.4	5.9	5–7.1
PLTS (10^9/L)	212.8	49	208	175–243

Hb = haemoglobin, WCC = white cell counts, PLTS = platelets.

2.5. Nanoparticle tracking analysis (NTA)

The MV enumeration by nanotracking was undertaken on a Nanosight NS500 instrument (Malvern instruments, Malvern, United Kingdom) equipped with a violet laser (405 nm). The instrument was standardized using 100, 200 and 400 nm beads (carboxylated polystyrene calibration particles, Izon Science UK). The samples were labeled with the Qdot 655 stain (Life Technologies/ThermoFischer Scientific, MA, USA) at a dilution of 1:100 and incubated at 37 °C for 1 h. The scatter (non-fluorescent) and fluorescent capture settings, including camera, focus and gain, were optimized so that particle tracks were clearly visible. When the capture was suboptimal (i.e. event capture rate either <20 or >200 tracks) further adjustments to dilutions were undertaken. Measurements were taken in triplicate for analysis using the Nanosight software (version 2.3 and 3.1) in scatter or fluorescent mode. A comparative analysis of the various techniques used in this study has been recently published [11].

2.6. Lipid and hormone profile

The lipid profile panel performed included Cholesterol (CHOL), triglycerides (TRIG), low density lipoprotein (LDL), high density lipoprotein (HDL) and total high density lipoprotein ratio (TOTAL HDLR). The hormone panel included testosterone (TESTO), luteinizing hormone (LH), follicle stimulating hormone (FSH) and progesterone (PROG). These tests were performed on the UniCel® DxI 800 Access® Immunoassay (Beckman Coulter Inc., CA, USA) clinical chemistry analyzer using manufacturer's instructions.

2.7. Small RNA quantitation

MV were pelleted from 1.2 ml of plasma by centrifugation at 21000 xg for 60 min. Total RNA was extracted using a slurry-based Norgen plasma/serum circulating RNA extraction kit (Norgen Biotek, ON, Canada) according to the manufacturer's instructions. The final volume of 100 μl of RNA containing eluate from each sample was vacuum concentrated to a volume of approximately 7 μl. The small RNA including miRNA content was evaluated using an Agilent 2100 bioanalyzer (Agilent technologies, CA, USA) using a dedicated small RNA chip. The results were analyzed using the bioanalyzer software for small RNA analysis.

Table 2
The median and IQ range of the MV parameters in the cohort of 108 subjects.

Parameter	Median	IQR	Mean	SD	SE of mean
CD41 MV/μl	1000.0	313.3–2770	3431.0	8154.0	784.7
CD105 MV/μl	391.0	227.3–3123	2339.0	5983.0	575.7
CD235 MV/μl	630.0	430.3–204	2501.0	5597.0	538.6
TF MV/μl	719.5	385.3–2193	2510.0	5476.0	527.0
AnnV MV/μl	266.0	97.75–1089	5714.0	22,490.0	2164.0
Scatter NTA ×10^7/μl	10.5	6–32.5	32.6	75.5	7.3
Fluor NTA ×10^7/μl	6.0	2–10.0	9.4	15.3	1.5
XaCT ng/ml	750.0	588–988.0	824.6	298.7	29.0
ETP RFU/min	1827.0	1324–2162.0	1790.0	553.6	54.3

2.8. Ethical and statistical considerations

The study was carried out in accordance with the National Statement on Ethical Conduct in Research Involving Humans (Australia). It was reviewed and approved by the Hunter New England Area Research as well as the University of Newcastle Ethics Committees. Written informed consent was obtained from each participant on this study.

The statistical analysis was carried out by SPSS (IBM Analytics, NY USA) and some graphical representations by Prism 6 (GraphPad, OK USA). The variables in the data set were evaluated for normality of distribution, data normalized by log transformation where appropriate and nonparametric testing was employed throughout for analysis as most variables showed a non-normal distribution. The comparative analysis was performed on log transformed represented as ranked data.

Differences between the groups were tested by the Mann-Whitney *U* test or by a Kruskal-Wallis followed by Dunn's multiple comparison where appropriate (significance at a p-value <0.05). Regression analysis

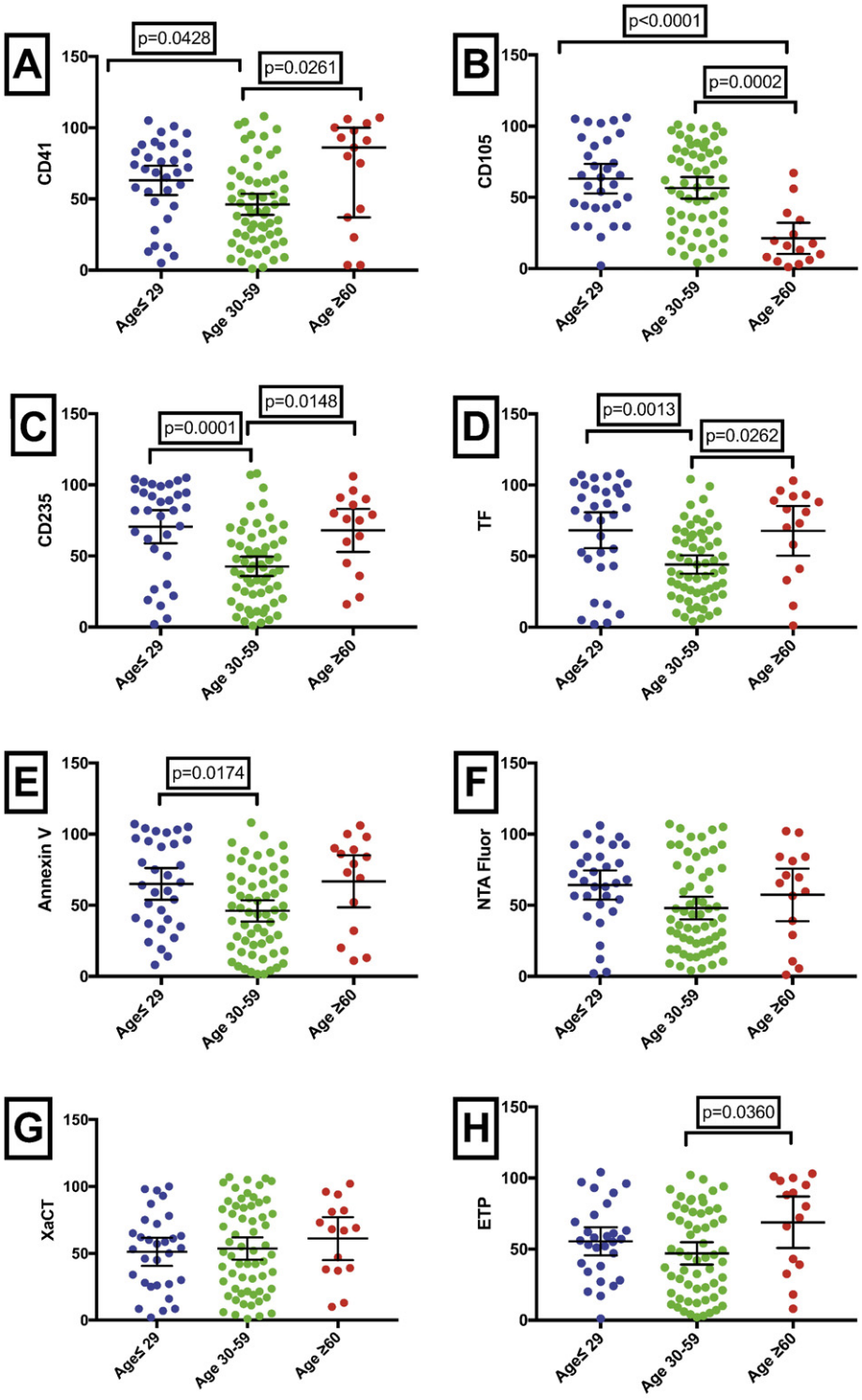

Fig. 1. MV differences by age: the layout with panels showing differences between the flow cytometry and ETP assays where significant. The mean ranks with 95% confidence intervals are depicted.

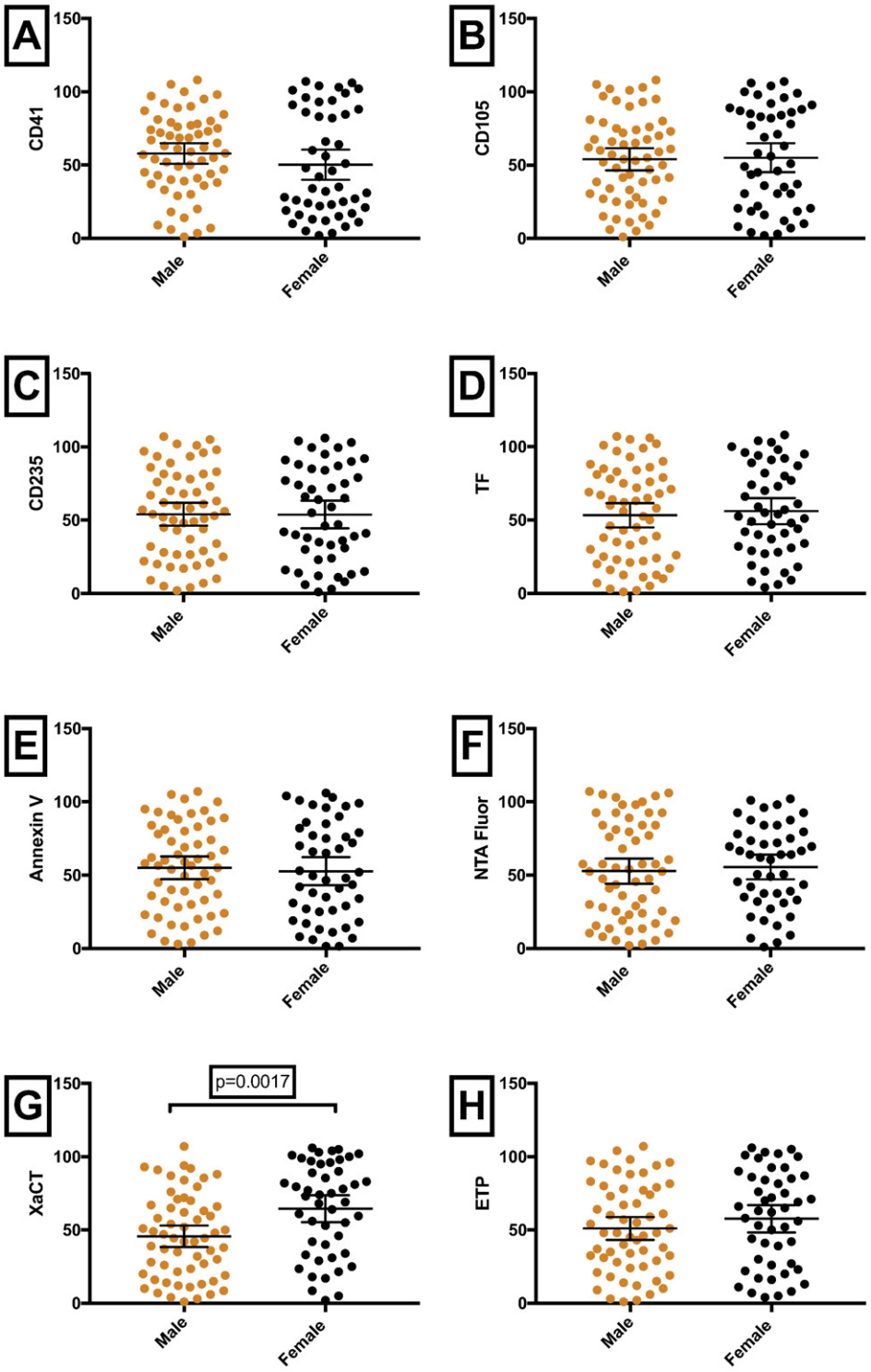

Fig. 2. MV differences by gender: the layout with panels showing differences by gender for MV determined by flow cytometry, functional assays, and by NTA. The mean ranks with 95% confidence intervals are depicted.[1]

after appropriate assumption checks was used to test the effect of independent variables in a predictive model. Correlations were performed using non-parametric tests (Spearman rank order correlations, r_s). A further analysis for significance at $p < 0.01$ and $p < 0.001$ was also undertaken inorder to correct for multiple hypotheses testing.

3. Results

A total of subjects 143 were finally analyzed (12 samples were excluded as they did not meet inclusion criteria) which included 80 males and 63 females. A cohort of 108 samples was available for the full range of tests with an additional 35 samples available for small/miRNA analysis. The baseline results for the full blood counts, age and BMI for the 108 samples are shown in Table 1. There were 60 males and 48 females in this cohort. The mean age was 40 years (range 16–

[1] Fluor is fluorescent.

73). This cohort was analyzed in three age groups of ≤29 (n = 32, designated group 1), 30–59 (n = 61, group 2) and ≥60 years (n = 15, group 3). The full blood count, gender and BMI was not significantly different between the three groups (data not shown).

The median values with interquartile range (IQR), mean as well as standard deviation (SD), standard error (SE) of mean and 95% confidence intervals (CI) for individual MV subsets by flow cytometry, total MV quantitation by NTA analysis, and functional procoagulant potential by XaCT and thrombin generation (ETP) is presented in Table 2. CD41 (platelet) MV constituted the largest fraction which was followed by CD235 (red cell) MV. Both TF and PS (as measured by annexin V binding) expressing MV constituted a significant proportion of the MV.

3.1. Age and MV level/function

Differences in MV fractions and functions between age groups are illustrated graphically in Fig. 1. Younger (group 1) and older (group 3) subjects tended to have higher levels of MV when compared to those in middle age (group 2), with significant differences in levels of CD41 (platelet), CD235 (red cell), TF and PS expressing MV between groups 1–2 and groups 2–3 respectively. In contrast, CD105 (endothelial) MV showed a decreasing trend with increasing age whereas ETP showed a higher level in older (group 3) compared to middle aged (group 2) subjects. There were no differences between the groups by XaCT test or levels as measured by NTA. However, it was observed the peak sizes of MV were variable between the age groups (Supplementary figure).

3.2. Gender and MV level/function

The median levels of CD41, CD105, CD235, TF and PS expressing MV, as quantified by flow cytometry, were generally similar or non-significantly lower in females compared to males (Fig. 2). There was no statistical difference between the two genders for any of the flow based MV.

In contrast, both functional assays demonstrated higher procoagulant activity levels in females and this was statistically significant for the XaCT test (p = 0.002). On the other hand, the NTA results by fluorescent mode did not vary significantly between the genders (Fig. 2).

Gender differences in MV parameters were also analyzed within each age group. In group 3, procoagulant activity measured by XaCT test was higher in females but significant differences were not observed for other analyses. These results are shown in the Supplementary Table 1.

To assess hormonal influences on MV parameters, a separate analysis was performed on females grouped into pre- and post-menopausal age groups (≦55 years vs >55 years, respectively) as shown in Table 3. The only difference observed was a lower number of CD105 MV in those above the age of 55 years.

3.3. Smoking status and MV level/function

There were 21 smokers and 87 non-smokers in the cohort. The CD41 platelet, TF and PS positive MV, by flow cytometry, were significantly lower in smokers (p = 0.029, <0.0001 and <0.0001 respectively) whilst there was no difference in levels of CD105 and CD235 MV (Fig. 3). Though ETP was also lower in smokers it did not reach statistical significance (p = 0.085). The total numbers of MV scatter events measured by NTA was higher, but there was no statistically significant difference in these, the size or the number of NTA fluorescent events between smokers and non-smokers (data shown for fluorescent NTA).

3.4. Multivariate regression analysis of association for prediction of MV levels by age, age group, full blood counts, gender and smoking status

A standard regression analysis was performed to identify the best independent predictors of each MV parameter as measured by flow cytometry, functional assays and NTA. The key results are summarized in Table 4. There was a trend for CD41, CD105, CD235 and TF to be negatively associated with age. Smoking status as an independent variable was predictive for CD235 and TF expressing MV. It was also negatively associated with CD105, annexin V and ETP. In addition, platelet counts positively predicted for ETP and there was a trend to significance for association with age. Gender was the only variable predictive for XaCT test results. A regression analysis with the lipid and hormone profile could not be undertaken due to collinearity issues and instead a correlation study is presented in the following section.

Table 3
Comparison of females ≤55 years and >55 years in relation to hormones and MV level/function.

Female	≤55 years (n = 36)		>55 years (n = 11)		
Variable	Median	IQR	Median	IQR	p value
LH[a]	5.8	2.9–15.2	27.2	20.5–30.1	<0.001
FSH[a]	6.4	3.3–12.9	65.0	50.1–92.6	<0.001
OESTR[a]	230.0	100–474.5	100.0	99–100	<0.001
PROGES[a]	2.9	1.4–9.4	0.9	0.5–1.6	0.003
CD41 MV/μl	436.3	267.4–2885	2297.7	197.9–13,790.8	0.28
CD105 MV/μl	2858.0	227–3531.2	191.4	123–246.2	<0.001
CD235 MV/μl	610.4	433.6–3131.9	658.8	359.6–2694.7	0.80
TF MV/μl	624.90	394.6–2155.1	1190.4	534.5–3943.5	0.38
AnnV MV/μl	191.5	98.7–816.3	234.1	42.9–1747.8	0.93
Scatter NTA $\times 10^7$/μl	8.0	2.9–16	867.9	11.2	0.38
Fluor NTA $\times 10^7$/μl	5.0	2.7–8	1821.5	8.6	0.12
XaCT ng/ml	867.9	623.1–1246.7	918	680.7–1004.7	0.93
ETP RFU/min	1821.5	1284.3–2121.5	2115.5	1692.3–2583.8	0.14

Kruskal-Wallis test for non-parametric variables, significance at p < 0.05.
[a] Mann-Whitney *U* test for parametric variables, significance at p < 0.05.

3.5. Correlation of MV level/function with lipid and hormone profile

Given the baseline physiological differences in the hormone and lipid profiles between the genders, males and females were analyzed separately. The CD41 platelet MV showed a weak to moderate negative correlation with BMI in males. PS expressing MV also showed a weak correlation with triglycerides whilst TF expressing MV correlated negatively with LH. The XaCT test correlated weakly with FSH (results in Supplemental Table 2). In females, CD235 red cell MV correlated with LDL, PS expressing MV with triglycerides whilst total MV enumeration by NTA in scatter mode correlated with triglycerides. Fluorescent NTA results negatively correlated with oestrogen and XacT functional assay positively with LH (results in Supplemental Table 3).

3.6. Small RNA and miRNA in MV

A second cohort of 35 samples was selected for miRNA evaluation, as the volume of sample was insufficient from the first cohort for miRNA extraction. This cohort had 35 subjects (males = 20, females = 15) with a mean age of 45.6 years (range 25–72). There were 9 subjects in age group 1, 13 in group 2 and 13 in group 3 with three smokers in the entire cohort.

The total amount of small, including miRNA, isolated from MV is presented in Table 5. The small RNA (5.7 ± 28.3 nt) and miRNA (22.6 ± 5.7 nt) levels were highly variable, but not significantly different across the three age groups. There was no difference in the quantity of small and miRNA in MV by gender. In multivariate regression analysis by age, gender and smoking, none of these variables were found to be predictive for miRNA in MV.

4. Discussion

This study was undertaken to characterize MV in a large cohort of normal subjects in relation to their age, gender, BMI, lipid and hormone

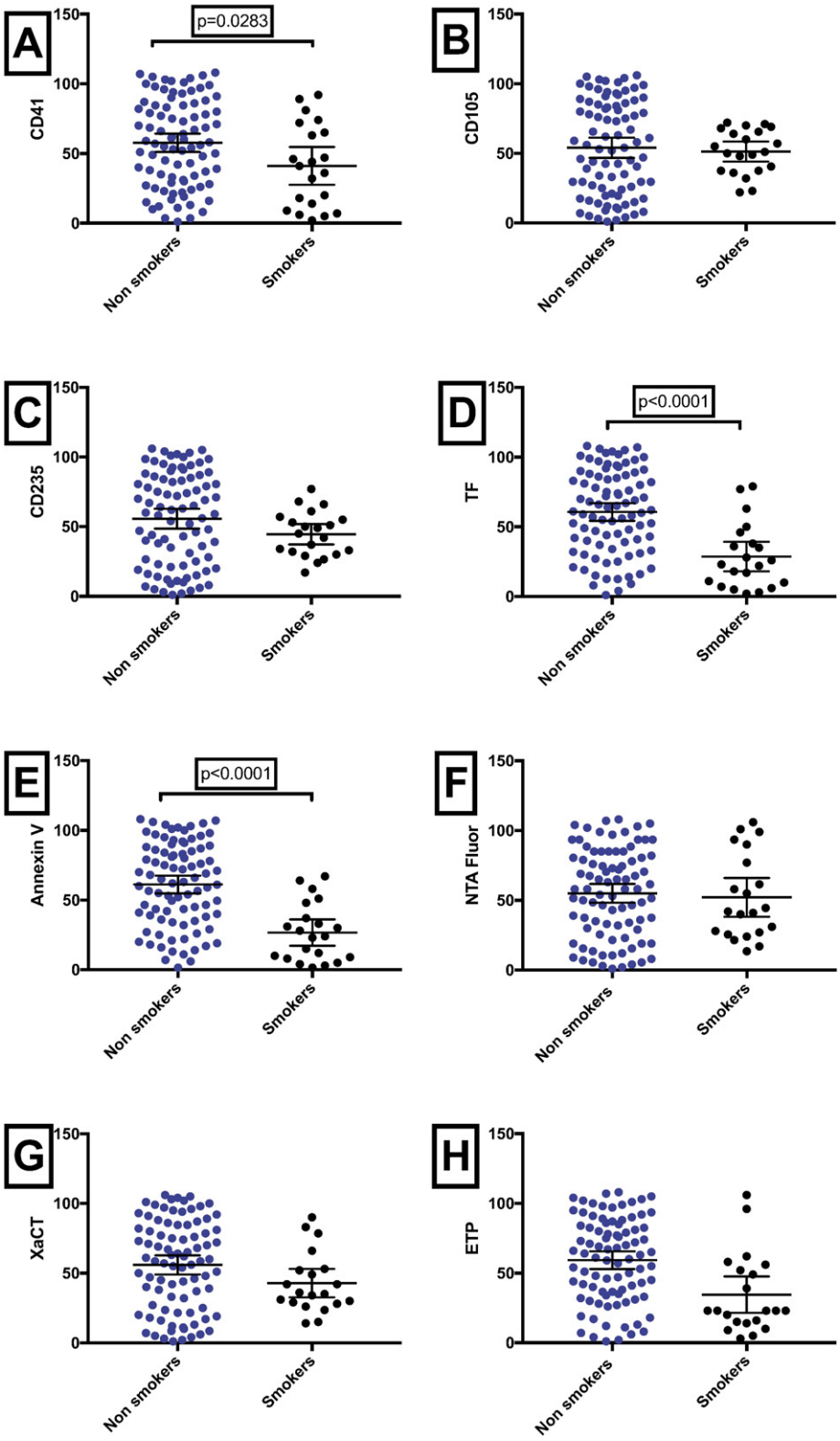

Fig. 3. MV differences by smoking status: the layout with panels showing differences by smoking status for MV determined by flow cytometry, functional assays and by NTA. The mean ranks with 95% confidence intervals are depicted.[1]

profile as well as smoking status. A range of enumeration techniques including flow cytometry, NTA and functional studies (ETP and XaCT) were performed in a cohort of 108 normal blood donors [9]. We also report on total small RNA and miRNA isolated from MV in an additional 35 healthy subjects. The evaluation of ranges in a normal healthy population is challenging. As MV are closely linked to pathology, the presence of subclinical pathology in an otherwise apparently normal healthy subject can still potentially impact the level and function of MV.

In general, it was observed that subjects aged 30–59 years had lower median levels and function of MV compared to those who were either

Table 4
Key independent predictors of MV parameters as determined by regression analysis.

Dependent variable	Independent variable	Beta coefficient	Standard error	Standardized Beta coefficient	t value	p value
CD41						
	Age	−0.006	0.003	0.23	−2.26	0.026
CD105						
	Age	−0.021	0.004	−0.52	−5.86	<0.001
	Smoking	−0.303	0.14	−0.19	−2.11	0.037
CD235						
	Age	−0.007	0.003	−0.22	−2.21	0.03
	Smoking	0.31	0.14	0.23	2.3	0.023
TF						
	Age	−0.009	0.003	0.26	2.8	0.006
	Smoking	0.50	0.13	−0.36	3.93	<0.001
AnnV						
	Smoking	−0.97	−0.22	−0.42	−4.46	<0.001
ETP						
	Age	0.002	0.001	0.17	1.75	0.083
	PLTS	0.001	0.0	0.27	2.70	0.008
	Smoking	−0.086	0.39	−2.17	−2.23	0.028
XaCT						
	Gender	0.12	0.041	0.42	2.96	0.004

Significance at $p < 0.05$, Hb – haemoglobin, WCC-white cell counts, PLTS-platelets.

younger or older, except for CD105 endothelial MV which showed a decreasing trend with age. These differences may explain some of differences in pathophysiology of disease seen across different age groups, although the exact reasons for the variation are not clear at this point. There is limited and inconsistent information on such trends in normal healthy subjects. In a previous study by Owen et al., although the procoagulant phospholipid by functional analysis did increase with age in subjects with venous thrombosis, the PS expressing MV levels by flow cytometry did not [13]. Jayachandran et al. reported that in subjects presenting to emergency department, they noticed a lower level of circulating MV in their clinically stable elderly subjects (>75 years) when compared to those <50 years [14].

Despite similar levels as measured by flow cytometry, female MV showed higher procoagulant function, as measured by XaCT test, compared to males. Our results showed that women over the age of 55 had lower median levels of CD105 expressing MV. This is consistent with other reports of lower endothelial MV levels in post-menopausal women [15]. Circulating levels of such endothelial derived MV can be used as a biomarker for endothelial function, and have been associated with subclinical abnormal coronary artery calcification [16]. The decrease in oestrogen experienced post menopause is known to contribute to a decline in vascular function, and thus we hypothesize that this may be linked to a change in levels of CD105 MV that would normally contribute to maintaining endothelial function.

The observation that the CD41 (platelet), TF and PS expressing MV were significantly lower in smokers compared to non-smokers in our cohort was an unexpected finding. In our study, there was also significantly lower function as analyzed by the XaCT test and a trend towards lower function with ETP. Not surprisingly, it was also unclear from published literature whether smokers 'do or do not show' an increased platelet function or increase in MV level/activity on cigarette exposure [17,18]. Despite conflicting results from various studies, the possibility that smoking influences MV levels must be at least considered in prospective studies that include both smokers and non-smokers.

Since the literature is discordant particularly with respect to relationship of age as well as smoking to MV levels or function we also undertook a regression analysis to evaluate whether age, gender, full blood counts or smoking status could predict MV levels as evaluated by flow, functional or NTA analysis. It was observed that CD41, CD105, CD235 TF were negatively predicted by age. Smoking status also independently positively predicted for CD235 red cell MV and TF MV, whilst CD105, PS expression and ETP predicted negatively.

The impact of BMI and lipid profile on MV level or function is not well studied. In our study, CD41 platelet MV correlated negatively with BMI in males. The CD105 endothelial MV correlated positively with cholesterol whilst PS expression correlated positively with LDL. In females, triglycerides correlated positively with the CD 41 platelet MV and PS expressing MV. Oestrogen correlated negatively with fluorescent NTA and positively with XaCT. There is little known on how lipid profiles influence or correlate with MV level and/or function even though it is well established that abnormal lipid ratios can lead to endovascular damage. In addition, in a previous report on obese subjects, elevated MV levels have been previously described [15].

We also performed NTA analysis and found no differences in MV by scatter or fluorescent modes with respect to age, gender or smoking status. Fluorescence based NTA has potential to provide information on MV of small sizes by tagging with quantum dots, which have a peak emission at 605 nm and a diameter of ~15 nm, are small enough to fit into the vesicles as small as 24 nm [3]. This is particularly useful in identifying vesicles of biological origin by virtue of high signal to noise ratio in the fluorescent mode, however, we did not find major differences in the levels of the healthy cohort using this mode. There were differences in the peak sizes of the vesicles between the age groups; the significance of which is not evident. We did not use a non-optical method such as resistive pulse sensing (RPS) which may have provided further information given it's sensitivity to both exosomes and smaller MV [19,20]. Even though NTA captures a wider range of vesicle size, functional assays and flow cytometric evaluation provide more comprehensive analysis of the function and the cell of origin of MV [3,8].

One drawback of our study is the smaller number of subjects in subgroup analysis such as gender by age group analysis (as provided in Supplement Table 1). As the techniques for well-established procedures like flow cytometry evolve, recommendations may change in the approach to measuring MV. A number of pre- analytical and analytical variables for each of these techniques can contribute to variability – for e.g. in flow cytometry the clone of the antibodies, gating strategy, swarm effect and type of flow cytometer used may all influence the results in varying proportion [21,22]. Freeze thawing and centrifugation steps can also influence MV levels. As newer techniques, such as NTA or RPS become more widely available, there may be increased uptake depending on their adaptability and functional utility.

There is now evidence that miRNA in MV can transfer information which can modulate other cells and tissues [23]. In this study, we report that the quantity of small RNA and miRNA from circulating MV in healthy subjects show a wide range of distribution but do not specifically vary by age, gender or smoking status. Depending on the quantity and type of miRNA contained, the potential to transfer information via MV with paracrine or autocrine impact may vary.

In conclusion, a number of pre-analytical and analytical variables for each of these techniques can contribute to variability between studies and laboratories [8]. However, even within a well-controlled environment, the likelihood that normal biological variability based on demographic or other individual factors, results in high baseline population variation in MV must be considered. It is therefore critically important to reflect on and consider these factors in a clinical study involving the measurement of MV for pathogenic potential.

Table 5
Distribution of total small RNA and miRNA isolated from MV in 35 healthy normal subjects.

	Small RNA		miRNA	
	Size (nt)	Conc (pg/μl)	Size (nt)	Conc (pg/μl)
Mean	51.74	403.49	22.6	121.68
Median	59	377.2	24	91
Std. deviation	28.29	252.32	5.72	97.81
Range	4–101	14.1–1010.9	0.1–67	0.1–338.2

nt = nucleotides.

Conflict of interest

None.

Contribution of each author

Project overview, supervision and analysis: AKE, MS and LL; experimental work: AKE, AA and ADC; references review and tables: AA and AKE; manuscript editing: all authors.

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Evaluating the normal variation in MV based on age, gender and other factors set the stage for investigating its role in diseases in the following chapter.

CHAPTER 5

MICROVESICLES IN DISEASE

5.1 General overview

Microvesicles exert a variety of effects and are dependent on the tissue or cell of origin. These effects of the MV are likely dependent on the surface antigens expressed and/or the cytoplasmic contents of the vesicle as shown in the flow chart below [figure 5(a)].

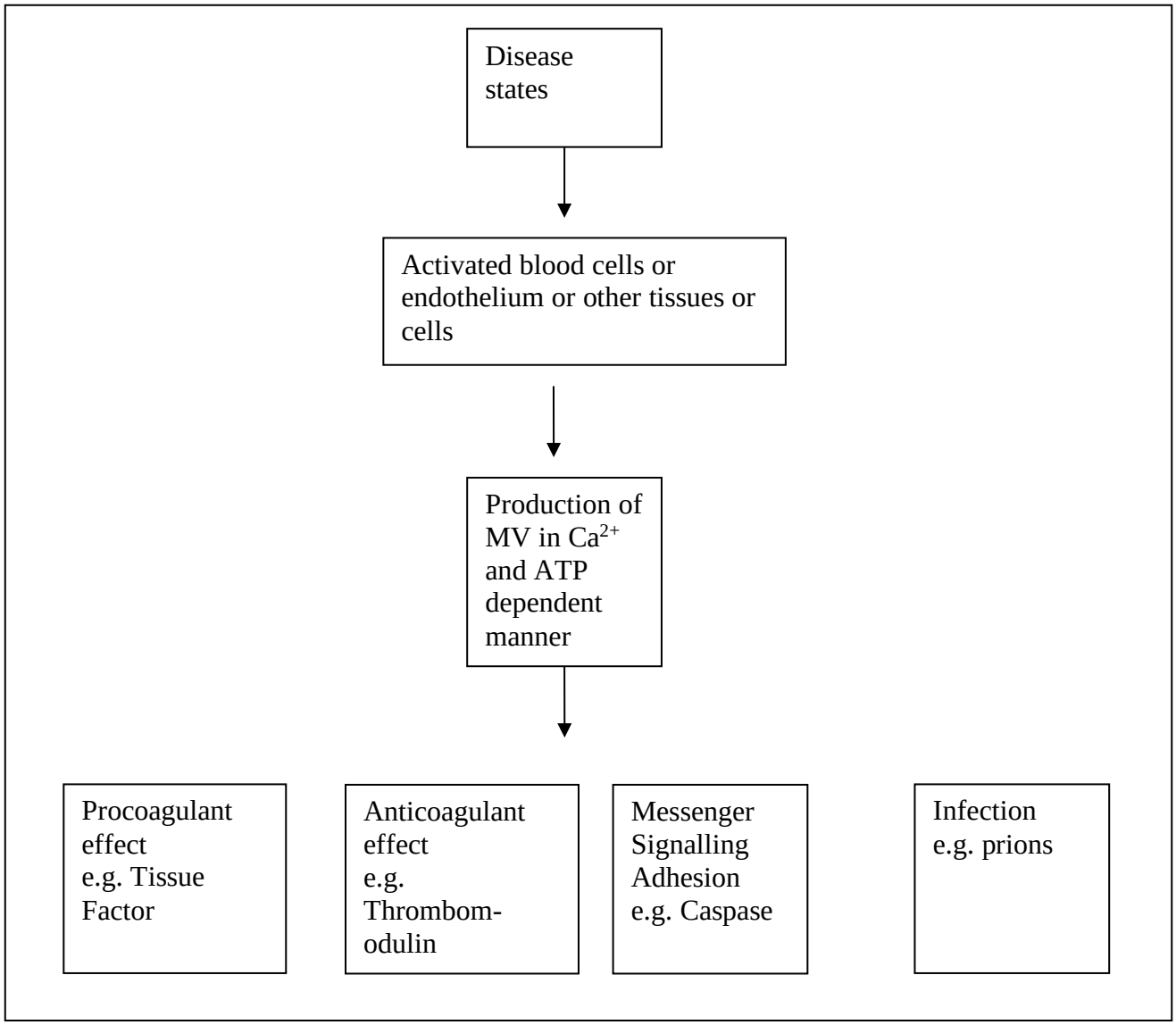

Figure 5(a). The mechanism of production and possible effects of MV. Adapted from reference (Enjeti et al, appendix paper 2).[17]

There are a number of conditions associated with elevated MV [see table 5(i) below], many of these are also associated with an increased risk of thrombosis.

Table 5(i). Conditions associated with elevated circulating MV. Reproduced from reference.[17]

System/ Condition	**Associated pathology**
Cardiovascular disease	Hypertension Myocardial infarct/angina Stroke Diabetes Thromboembolism
Myeloproliferative Disorder	Polycythemia vera Essential Thrombocytosis Myelofibrosis
Thrombotic Microangiopathies	Thrombotic thrombocytopaenic purpura Heparin induced thrombocytopaenia Pre-eclampsia of pregnancy
Antiphospholipid Syndrome	Antiphopholipid antibody syndrome Systemic lupus erythematosis
Cancer	Metastatic solid tumours Chemotherapy induced MV Neoangiogenesis

PAPER 4 (Book Chapter):

Enjeti AK, Seldon M. Microparticles : Role in Haemostasis and Venous Thromboembolism (ed 2012): InTech; 2012.

Learning points

MV play an important role in vascular biology and coagulation. Raised MV levels are associated in sevreal prothrombotic states and reduced in some rare bleeding conditions.

Note : the publications are embedded as pdf and retain their original page numbers as in the manuscript.

1

Microparticles: Role in Haemostasis and Venous Thromboembolism

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1. Introduction

Microparticles (MP) are small membrane bound vesicles which have been described in circulation. They are derived from a variety of cells by an active process of shedding. They are bound by plasma membrane, are anucleate but may contain DNA or RNA and may be virtually derived from any cell (Ahn 2005, Mause, *et al*, Porto, *et al*). The majority of the microparticles in blood are derived from platelets. Previously considered as cell debris they are now regarded as vectors for transfer of biological information. The MP production is thought to reflect a balance between cell stimulation, proliferation and death. Based on their potential function and pathophysiologic effect, MP are thought to be physiological or patholological. MP play a role in normal haemostasis and abnormal amplification of MP production leading to a pathological state (Meziani, *et al*). For example, excessive MP from platelets may contribute to thrombosis (Siljander, *et al* 1996). Their role in vascular biology is being uncovered with increasing evidence for their role in venous thromboembolism. This chapter will explore the role of these MP in the physiology of haemostasis as well as pathology of thromboembolism. The final section will discuss the current state of art in the methods used to detect and measure MP.

1.1 Definition of a microparticle

Microparticles are submicron (<1.0μm) membrane bound circulating vesicles. Although anucleate, usually express cell surface antigen specific to the cell of origin, they may contain DNA or RNA and be virtually derived from any cell (Freyssinet 2003). The ISTH (International Society of Thrombosis and Haemostasis) vascular biology subcommittee defined these particles as being between 0.1-1.0 μm (SSCMembers Aug 2005). However, several other nanoscale techniques have demonstrated that particles <0.1 μm may also need to be considered as MP (Yuana, *et al*). Indeed, size range of MP is contentious with larger MP likely overlapping with small platelets and the smallest MP with exosomes (Gyorgy, *et al*, Jy, *et al*, Lawrie, *et al* 2009). Several factors may cause the production of MP from cells such as activation, complement mediated lysis, shearing stress, oxidative injury and active vesiculation (Horstman, *et al* 2004). The MP bear at least some surface characteristics of the parent cell and they differ from exosomes (0.03-0.1μm), which originate through the exocytosis of endocytic multivesicular bodies and play a role in antigen presentation (Freyssinet and Dignat-George 2005, Horstman, *et al* 2004, Horstman, *et al* 2007).

Cellular source of MPs	Marker	Proportion in circulation
Platelets	CD61 (GPIIIa) CD63 CD62p (P-selectin) CD41	80-90%
Leucocytes	CD45	<5%
Erythrocytes	Glycophorin A	5-10%
T helper cells	CD4	<1%
T cytotoxic cells	CD8	<1%
B cells	CD20	<1%
Monocytes/ macrophages	CD14	<5%
Endothelial cells	CD62e (E-selectin)	<5%

Table 1. Microparticle source, surface antigen expression and proportion in circulation (Enjeti, *et al* 2007, Siljander).

2. Microparticles: Production and role in haemostasis

2.1 How are microparticles produced?

Microparticles are thought to be produced by an active process of vesiculation or shedding from the cell surface and utilizing ATP in the process. Various enzymes involved in the production of MP have been studied. The balance of several enzymes regulating membrane homeostasis is believed to be key in the production of MP. An inward aminophospholipid enzyme 'translocase' or 'flippase'and an outward enzyme 'floppase' have been postulated to maintain the dynamic symmetrical state of the phophoslipid bilayer membrane (Diaz and Schroit 1996, Montoro-Garcia, *et al*, Morel, *et al*). In a resting membrane the flippase enzyme is more active thereby ensuring that phosphotidyl serine (PS) is at the inner membrane.The activation of phospholipid nonspecific enzyme known as 'scramblase' is said to be responsible for disruption of membrane asymmetry and several mechanisms participating in the regulation of the transmembrane migration of phosphatidylserine (PS) in activated cells lead to microparticle shedding (Diaz and Schroit 1996, Enjeti, *et al* 2008, Morel, *et al* 2006). After stimulation, calcium is released from intracellular stores. Calcium depletion induces the activation of store-operated calcium entry (SOCE) through channels in the plasma membrane and this process is thought to be regulated by transient receptor potential channel (TRPC) proteins (Diaz and Schroit 1996, Montoro-Garcia, *et al*). The transverse redistribution of PS is under the control of SOCE. Several other process such as Raft integrity, cytoskeleton organization and MAP kinase pathway (Ras-ERK) are also involved in membrane remodelling (Diaz and Schroit 1996, Montoro-Garcia, *et al*, Morel, *et al* 2006). Microparticles typically have phosphotidyl serine on the outer surface (although PS negative MP have also been recently described) and ABCA1, a member of the ATP-binding cassette family of transporters, is a potential candidate for the transport of PS to the surface (Diaz and Schroit 1996, Morel, *et al* 2006).

2.2 Role of MP in coagulation and haemostasis

Normal coagulation is a complex process triggered by endothelial damage and exposure of tissue factor and collagen which initiates a platelet plug formation at the site of injury. This

leads to activation of a cascade of enzymes, which forms a fibrin clot. Microparticles of different cell origin could play a role in fibrin clot formation, enhance platelet leukocyte interactions and influence other plasma proteins such as von Willebrand's factor. Given that platelet MP constitute the majority of the circulating MP, they are considered an important effector of the haemostatic process (Morel, *et al* 2006). Some MP have also been described to carry molecules with anticoagulant function on their surface (Freyssinet 2003). The balance of pro and anticoagulant bearing MP in the endovascular milieu is likely to influence the propensity to bleed or clot in a particular patient.

Type of MP	**Example of surface marker on MP**
Procoagulant	von Willebrand's Factor Tissue Factor Platelet Factor 3 activity
Anti-coagulant	Tissue Factor Pathway inhibitor Protein C/S Thrombomodulin

Table 2. The possible pro and anticoagulant markers on the surface of microparticles (Enjeti, *et al* 2007, Morel, *et al* 2006).

2.3 Platelet MP

2.3.1 Platelet MP and coagulation

Traditionally, platelets major function was thought to be due to their aggregability and ability to plug damaged endothelium and capillary vessels. More recently, they are thought to form an important substrate for the coagulation pathway with their membrane providing the surface for the formation of the prothrombinase complex (comprising the Xa and Va complex). This enzyme complex leads to conversion of fibrinogen to fibrin which in combination with a variety of other factors leads to a stable clot at the site of injury. The presence of platelet microparticles at the site of blood vessel injury may contribute to this process by providing a large source of surface membrane for assembly of the enzymatic process. Indeed the exposure of phosphotidylserine at the site of thrombin generation increases the enzymatic catalyic effect by several hundred fold (Aleman). Platelets thus appear to have two major physiological roles for achieving haemostasis - form a platelet plug at the site of endothelial injury and generate microparticles which provide a surface for activation of the coagulation cascade leading to formation of the fibrin clot. The third possible role for the platelet MP could possibly be in maintaining the integrity of normal resting endothelium (Cambien, 2004). This area is still being actively explored. The role of MP in haemostasis is illustrated in figure 1.

Apart from procoagulant function MP could also be involved in anticoagulant activity. Microparticles with TFPI (tissue factor pathway inhibitor) and antithrombin activity have been described (Morel, *et al* 2006, Siljander). However, the anticoagulant MP have not been as extensively studied and it would be interesting to evaluate these MP - its association with pathologic conditions.

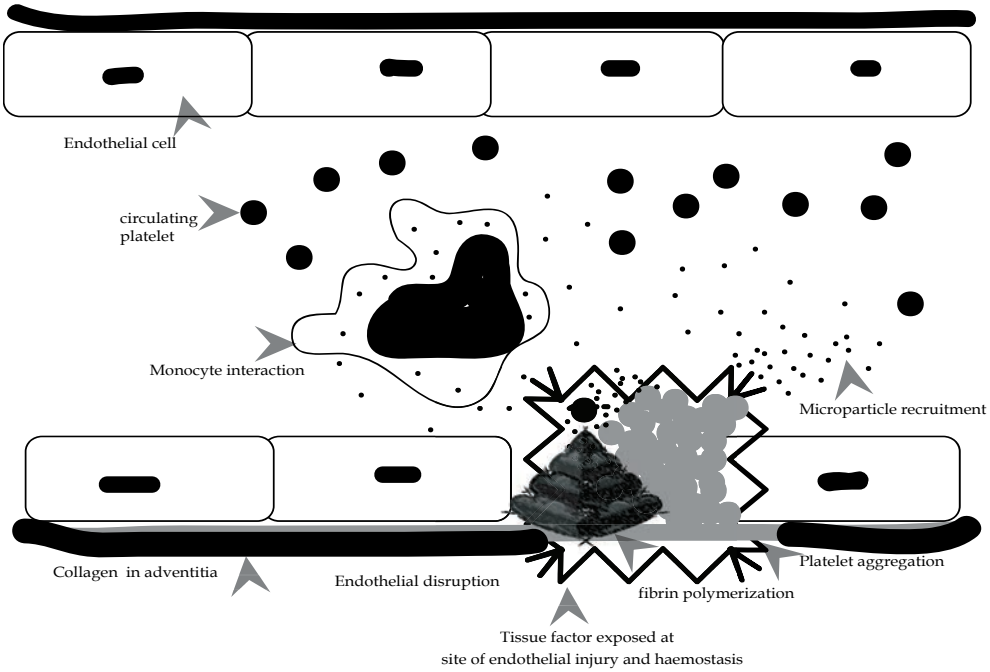

Fig. 1. The interaction of MP of platelet and monocyte origin being recruited in thrombus formation at site of endothelial injury.

2.3.2 Molecular interactions of Platelet MP

Platelet MP also bear a number of antigens such GPIIbIIIa, GPIa, von Willebrand's factor and arachidonic acid which may all be important effectors in the clotting mechanism. The understanding of the molecular mechanisms of haemostasis has now led to the thinking that coagulation can be described as an interaction between p-selection, tissue factor thrombin and microparticles (Furie and Furie 2004). P-selectin is an adhesion molecule expressed at the platelet endothelial interface which is thought to be critical for tissue factor activity and leukocyte adhesion in the thrombus (Myers, 2003). Some authors have even described P-selectin on microparticles, tissue factor and clotting proteins as being the molecular triad for coagulation (Polgar, 2005).

Another potential role of MP may be in the interaction of endothelium, von Willebrand's factor and platelets.The platelet derived microparticles could interact with the protease ADAMTS-13 (**A D**isintegrin **A**nd **M**etalloproteinase with **T**hrombo**S**pondin-1-like motifs, member **13** of this family of metalloprotease) , which regulates the activity of high molecular weight von Willebrand's factor. Increased microparticles in circulation could potentially compete in binding ADAMTS-13, reducing its interaction with the endothelium and influencing multimer cleavage (Jy, *et al* 2005). This may then contribute to the increased rates of thrombosis observed in these patients with thrombotic thrombocytopaenic purpura though the evidence for this process is very preliminary.

2.4 Tissue factor bearing MP

In an intact blood vessel tissue factor is usually restricted to adventitia and protected by the endothelial layer. However, small amounts of monocyte related tissue factor have been isolated in circulation (Key). The presence of tissue factor (TF) bearing microparticles, mainly derived from monocytes, in circulation has been shown to participate in initiation of fibrin polymerization (Eilertsen and Osterud 2004, Key). Although usually found to be in very small numbers in normal circulation, these increase dramatically at the site of injury. The interaction between tissue factor bearing MP and platelet MP is also of interest as there appears to be some evidence that they may be complementary in terms of thrombin generation potential (Key and Kwaan).

2.5 Modelling MP in thrombosis

The evidence for the involvement of these MP in its various physiological roles in haemostasis comes from the following models.

2.5.1 Cell based haemostasis model

The initial evidence for the role of MP in haemostasis comes from the cell based model. In this model plasma coagulation proteins are activated on the membrane surface after exposure to tissue factor. This leads to enzymatic cleavage of thrombin from prothrombin which ultimately converts fibrinogen to fibrin. This forms the fibrin clot and leads to haemostasis along with other components of the clot such as platelets and monocytes (Biro, *et al* 2003, Chirinos, *et al* 2005).

2.5.2 Live imaging model

Studies using intravital microscopy have shown that TF bearing MP derived from haemopoietic cells are incorporated into a thrombus. A laser injury model using the cremaster muscle arterioles of the mouse showed that MP participate in thrombosis (Falati, *et al* 2003). Although these studies visualize incorporation of TF bearing MP into the thrombus, it is not yet known if these MP are actually functional.

2.5.3 Animal models

These studies have involved introducing exogenous MP from patients or other source into animal models. In one such study MP from patients with acute coronary syndrome were introduced in to a rat model triggered venous thrombosis (Mallat, *et al* 2000). This study supports the role of TF bearing MP in promotion of VTE, However, the cellular sources of this TF has not been entirely clarified in other studies (Shantsila, *et al*).

2.5.4 Scott Syndrome

Scott Syndrome is an extremely rare hemorrhagic disorder characterized by bleeding diathesis (only three well documented cases of Scott syndrome have been reported to date) (Zwaal, *et al* 2004). The bleeding tendency is thought to be due to impaired procoagulant activity of stimulated platelets – the platelets being unable to expose anionic phospholipids and to shed procoagulant microparticles. The exposure of the aminophospholipids, mainly

phosphatidylserine, on surface of stimulated platelets or derived microparticles, is critical for the formation of enzyme complexes in the clotting process (Zwaal, *et al* 2004, Zwaal, *et al* 2005). Mutations involving the ABCA1 ATP transporter have been reported in this syndrome (Zwaal, *et al* 2004).

There are several other mechanisms by which MP influence the endovascular system. They may modulate endothelial function and carry proangiogeneic molecules (Lozito and Tuan). Recently MP bearing Sonic hedgehog have been shown modulate angiogenesis (Soleti, *et al* 2009, Soleti and Martinez 2009). They may also serve as novel carriers for transport of genetic material – such as mRNA or microRNA and these are currently areas of intense research (Rak).

3. Role in thrombosis

From their role in physiology of haemostasis it can be extrapolated that excess production of MP will lead to a pathological state. Indeed, increase in circulating MP have been described in a wide variety of states. The role of MP in various thrombotic states is discussed below.

3.1 Venous Thromboembolism (VTE)

3.1.1 Idiopathic VTE

Venous thromboembolism is the result of a complex interaction between the circulating proteins, cells/platelets and the endothelium (Collen and Hoylaerts 2005). There is no known provoking or identifiable precipitating factor in idiopathic VTE. A recent study looked at the interactions between the MP of various origin - platelet, endothelial and monocyte and endothelial derived MPs were found to be elevated in association with VTE. One report suggests that the combination of total circulating MP, P-selectin levels and D-dimer levels may help predict VTE (Rectenwald, *et al* 2005). This approach had a sensitivity of 73% indicating the need for further refinement for application in clinical practise.

In another larger investigation no association was found between levels of total circulating MP and risk of recurrent VTE (Ay, *et al* 2009). Interestingly, a study comparing patients with cancer who had VTE and those with idiopathic VTE found raised tissue factor bearing MP only in cancer patients (Thaler, *et al*). In another report, plasma levels of tissue factor MP were not raised in those with pulmonary embolism suggesting that that perhaps other subtypes of MP may have to be studied in more detail to explain the relationship found in experimental models (Garcia Rodriguez, *et al* 2010). Owen and co authors looked at the recurrence of VTE and found that the procoagulant activity but not number of MP was increased in cases of recurrence (Owen, *et al*).

The role of MP in predicting thrombosis in those with heritable thrombophilia has also been explored. It has been found that total circulating MP levels were increased in subjects with heterozygote factor V Leiden status but there was no difference between those who had had VTE and those without (Enjeti, *et al* 2010). This finding and other studies seems to suggest that although total microparticles have been shown to be increased in those with VTE or those prone for VTE, there appears to be no convincing data that MP help to predict or monitor VTE. However, in a recent study that investigated this issue further, looked at MP levels by a different approach by comparing percentiles of MP measured in a retrospective

case-control fashion. In those with circulating MP above the 90th percentile of the control population's distribution, a five fold increased risk was observed (Bucciarelli, 2011). They found that elevated MP were indeed an independent risk factor for VTE and this warrants a confirmation in a prospective cohort study.

The draw back of the studies in this area of VTE include the variability of type of MP studied, the techniques employed for measurement of MP and retrospective nature of investigations undertaken.

3.1.2 Immune related VTE

In contrast to idiopathic VTE, there is strong evidence for involvement of MP in thrombogeneticity in patients with underlying immune disorders. Important examples include antiphospholipid antibody syndrome and heparin induced thrombocytopaenia with thrombosis syndromes (Combes, *et al* 1999, Dignat-George, *et al* 2004, Walenga, *et al* 2000). Markedly elevated platelet derived MP have been desccribed in both clinical syndromes (Hughes, 2000). There is experimental evidence to suggest that circulating autoantibodies trigger the formation of excess MP contributing to the prothrombotic process in these patients. Circulating MP in these syndromes have been shown to expose GPIb,GPIIbIIIa, P-selectin and thrombospondin all of which help promote thrombosis (Jy, *et al* 2007).

3.1.3 Microparticles, VTE and cancer

In contrast to the above discussion for idiopathic VTE – thrombosis, cancer and microparticles seem to have a more definitive relationship. The MP are thought to reflect a balance between cell stimulation, proliferation and death which may be important in cancer related thrombosis. Cancer increases the risk of VTE by four fold and addition of chemotherapy further increases the risk by six to eight fold (Furie and Furie 2006). It is possible that circulating MP shed from cancer cells represent an indication for tumours to metastasize in the absence of any other clinical evidence for metastasis. A recent report states that platelet MP markedly stimulated the metastatic potential of 5 different cancer cell lines (Rak). It has also been shown that human tumor derived MP when injected into mice activated coagulation by virtue of their TF procoagulant activity (Thaler).

Procoagulant properties of tumor cell MP have been an area of intense study. A range of endothelial, monocyte and leukocyte MP along with tissue factor bearing MP appear to have a coagulant potential and have shown to be elevated in various such as cancers such as pancreatic, breast and prostate (Pilzer, *et al* 2005, Simak and Gelderman 2006).

A recent in vivo live microscopy mouse model with pancreatic cancer demonstrated that TF bearing MP released from the cancer cells entered circulation and participated in the thrombus formation at a distant site (Thomas, 2009).

The most important evidence for role of MP in VTE and cancer comes from clinical studies showing increased numbers and procoagulant activity of MP in cancer (Langer). Elevated levels of tissue factor bearing MP were associated with VTE events in those with advanced malignancy particularly pancreatic cancer. The microparticle levels in cancer patients also predicted the development of thrombosis, with the one year estimate of those with TF

bearing MP being about 34% (Thaler, 2011). In contrast those who did not develop thrombosis did not have a detectable level of tissue factor bearing microparticles.

3.1.4 Disease groups associated with venous or arterial thrombosis

There are a number of conditions associated with elevated MP. Most of these disease states are associated with an increased risk of thrombosis. They essentially seem to reflect the health and pathophysiology of the endovascular system. Table 3 below gives a list of conditions where they have been found to be elevated.

Condition	Specific example where MP were elevated (reference)
Cardiovascular disease	Hypertension (Boulanger) Myocardial infarct/angina (Nagy) (Exner, 2005) Stroke (Merten, 2004) Diabetes (Alkhatatbeh) Thromboembolism (Cimmino)
Myeloproliferative Disorder	Polycythemia vera (Duchemin) Essential Thrombocytosis (Villmow, 2002) Myelofibrosis (Villmow, 2002)
Thrombotic Microangiopathies	Thrombotic thrombocytopaenic purpura (Ahn, 2002) Pre-eclampsia of pregnancy (Aharon)
Autoimmune diseases	Antiphopholipid antibody syndrome (Combes, 1999;Dignat-George, 2004) Systemic lupus erythematosis (Nielsen; Pereira, 2006)
Cancer related	Metastatic solid tumours (Dass, 2007) Chemotherapy induced (Kim, 2002; Kim) Neoangiogenesis (Goon, 2006)

Table 3. List of conditions associated with thrombosis and elevated MPs in circulation.

3.2 Microparticles and atherothrombosis

The role of MP in promoting atherothrombosis has also been another area of study (Cimmino). In one report, shed membrane microparticles were seen to be produced in human atherosclerotic plaques and were a critical determinant of thrombogenecity after plaque rupture (Mallat, 1999). The apoptosis occurring after plaque disruption or rupture was closely associated with TF expression on cell membranes leading to thrombogenecity. These MP were observed to express phosphotidylserine and some expressed CD11a which is an adhesion molecule (Martinez, 2005) (Morel, 2006). Given the links between inflammation and thrombosis, the emerging role of MP in atherothrombosis is not surprising (McGregor, 2006; Meerarani, 2007).

4. Measuring microparticles

There are several approaches to detection and measurement of MP. The methods are usually based on the ability of the assay to either enumerate or assess functional activity of the MP.

4.1 Functional assays

Most of the assays under this section relate to either the prothrombotic function of MP or measuring the phospholipid content of MP. This can be done in the liquid phase e.g. a clot based assay such as the XACT test or by estimation of prothrombinase activity using an ELISA (Exner, 2003). The advantages of these approaches are that they provide an indication of the procoagulant activity of MP. The drawback is that the cell of origin for the MP cannot be determined.

4.2 Quantitative assays

Flow cytometry is the most widely employed quantitative technique. The gating of small particles continues to be a challenge but flow cytomtery continues to be the only robust technique which can demonstrate the cell of origin for the MP. This is an important asset of flow cytometry. However, there is significant variability amongst flow cytometers and the ISTH subcommittee on vascular biology recently conducted a workshop on standardization of MP by flow cytometry (Lacroix). It remains a popular approach for detection of MP for the following reasons:1)Rapid turn around time 2)Both fresh and frozen specimens may be used 3)The expression of two or more antigens on the MP may be simultaneously demonstrated 4)Easy method for quantification using commercial beads.

However it has the following drawbacks: 1) The detection of particles less than 0.3μm is difficult by flow cytometry as the detection is limited by particle size in the same order of magnitude wavelength of the laser (about 488 nm 2) Different machines have different sensitivities 3) It is difficult to automate 4) Centrifugation speeds for sample processing are variable and not standardized (Freyssinet, 2005). Several new approaches to flow cytometry include using impedance flow cytometry and using Raman microspectrophotometry effect to cover the size and particle discrimination issues (Ayers, 2011).

The capture of MP into immobilized annexin V or cell specific antibodies using an ELISA based assay have ben the other major approaches (Enjeti, 2007). Solid phase assays have the advantage of picking up microparticles irrespective of size. However interference of soluble antigens, variable quality of antibodies used for antigen capture and non-exclusion of microsomes are some of the disadvantages.

4.3 Nanoscale and newer technologies

In the recent years there has been an adaptation of nanoscale technologies such as atomic force microscopy and nanoparticle measurement techniques. These methods claim to accurately measure particles in the nanoscale size range (Yuana). For example , one such nanoscale technique uses the brownian motion of these small particles to detect and measure them (Harrison, 2009). These methods are expensive, intensive to perform and not yet widely available (Lawrie, 2009). Moreover, the clinical utility of such techniques is not yet established. Recently a proteomic approach to analysis of MP has been described,

however, the clinical utility of this approach is also as yet unkown (Howes ; Ramacciotti). Automated devices to analyse MP are also being developed (Wagner, 2010).

4.4 Measuring microparticles: Future directions

There are several outstanding issues such as standardization of preanalytical and analytical variables as well as integration of the various approaches in measuring MP. Several novel approaches are now being considered. 'Megamix beads' is novel approach to standardizing of gating of microparticles using flow cytometry. It uses a mix of a 0.9um and 0.3um sized beads to try and capture all events within the gate set by the beads (Robert, 2009 ;Robert, 2011). One of the problems of using this approach is the lack of linearity in the relationship between the size of beads and forward sctatter at that particle size. A recent commercially available nanoscale technology known as 'Nanosight' has incorporated antibody tagging of small particles for accurate identification and counting in this size range (Harrison, 2009).

5. Conclusions

Utility of Measuring MP in venous thromboembolism is yet to be fully established . The case for measuring MP in cancer related VTE is perhaps stronger. There are three areas within which the potential for detecting and measuring MP with respect to venous thromboembolism may be relevant.

5.1 Diagnostic

The evidence for using measurement of MP in a diagnostic setting is limited. The studies so far have shown variable results depending on whether TF bearing MP, functional activity or total MP were measured. With respect to VTE MP have been assessed in the paradigm of VTE, diagnosis in a small pilot study where it was shown that D-dimer, P-selectin and total MP levels predicted thrombosis as demonstrated on Doppler ultrasound (Ramacciotti; Rectenwald, 2005). The role of MP in diagnosis of VTE warrants confirmation in prospective cohort studies. The standardization of measurement of MP will go a long way in ensuring comparability of such studies.

5.2 Prognostic

The potential for MP as a prognostic tool is dependent on reliable, reproducible and easily available tools to measure microparticles. There is emerging data that MP may predict VTE in cancer patients and may be able to provide prognostic information in several other conditions.

5.3 Therapeutic

An interesting dimension to this area is the approach to use or modify MP for therapeutic benefit. The possibility of bioengineered and/or harvested membrane microparticles in tissue repair or angiogenesis is being investigated (Soleti, 2009). The MP are also being studied as a drug delivery tool (Benameur, 2009). Microparticles could potentially be specifically targeted to reduce or prevent thrombotic complications or end organ damage (Myers, 2005). This is an promising and exciting new area for researchers and clinicians working in this area.

Microparticles have therefore emerged as key role players in vascular biology and pathophysiology of thrombosis. They remain an important research tool and their clinical applications are being actively investigated with potential to be applied in diagnostic, prognostic and therapeutic arenas. They are small yet powerful effectors for the pathophysiology of the endovascular system.

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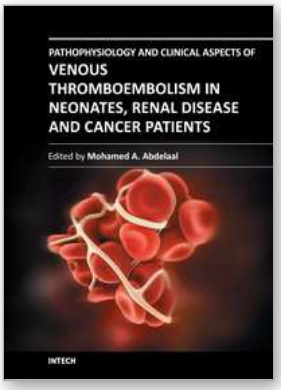

Pathophysiology and Clinical Aspects of Venous Thromboembolism in Neonates, Renal Disease and Cancer Patients

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Venous Thromboembolism remains a major health challenge in many countries because of the morbidity and mortality it inflicts, mainly in hospitalized patients. This book, with contributions from distinguished experts in the field, depicts some hot aspects on aetilogics of VTE, the disease burden in neonates, renal disease and cancer patients as well as issues relevant to prophylaxis and the concept of VTE as patient injury content.

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5.2 Microvesicles in pathological thrombosis - factor V Leiden

Levels of MV are reported to be higher in patients with venous thrombosis as well as arterial thrombosis when compared to healthy subjects.[17] Factor V Leiden (FVL; named after the city where it was first described) refers to the coagulation protein factor V containing a point mutation that confers an increased risk of venous thrombosis. A missense base substitution in exon 10 results an amino acid change from arginine to glutamine.[93] Normally, factor V is regulated by an anticoagulant called protein C. The FVL with the nucleotide change results in a protein that is resistant to the action of protein C and therefore increases thrombotic risk.[93]

The usual rate of venous thromboembolism in the general population is 1 in 1000 per year. A heterozygote FVL mutation increases that risk of thrombosis to between 4 to 8 in 1,000 whilst in homozygotes, the risk is as high as 80 in 1,000.[94] Clearly, not everyone with the mutation (either hetero or homozygote) experience a clot during their lifetime.[94,95] There are no known clinical or biomarkers that can potentially predict this risk. Given the emerging role of MV in venous thrombosis, we postulated that quantifying MV may be important in assessing the thrombogenic profile of a person carrying the factor V Leiden mutation (both heterozygote and homozygote).

Aim 3:

To undertake a comprehensive analysis of circulating MV in homozygote and heterozygote carriers of Factor V Leiden and compare with normal healthy subjects.

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Key learning and reflections

MV are elevated in a wide range of conditions associated with pathological thrombosis. Circulating MV levels are raised in factor V Leiden heterozygote mutation carriers. No definite difference in those with and those without thrombosis was observed.

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Brief Communication

Circulating microparticles are elevated in carriers of Factor V Leiden ☆

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ABSTRACT

Introduction: Microparticles (MP) are small membrane bound cellular particles that play an important role in thrombosis. This study was carried out to evaluate if increased numbers or procoagulant potential of circulating MP contribute to the heterogeneity in occurrence of thrombosis in heterozygotes carrying Factor V Leiden (FVL) mutation.

Methods: Levels of circulating platelet (CD41a), endothelial (CD62e) as well as leukocyte (CD45) derived MP from 45 FVL heterozygous individuals were enumerated by flow cytometry and compared with normal controls. Functional studies included enzyme linked immunoassay based prothrombinase activity (ELISA) and modified dilute Russell Viper venom test (DRVVT).

Results: Circulating MP were significantly higher in the FVL cohort compared to the controls (median = 2100 vs. 1508 MP/μl, respectively $p = 0.0021$).All subsets of MP (platelet, endothelial and leukocyte) were significantly elevated in the FVL group, the most striking disparity seen in the number of CD45 positive leukocyte MP. Despite the differences in the number of MP between the controls and FVL cohorts, there was no significant difference in the prothrombinase activity recorded by the ELISA (2.0 vs 2.4 PS equivalents; $p = 0.7374$) or clotting time assessed by the DRVVT (47 vs 46 sec, $p = 0.8118$). When the FVL cohort was considered alone there was no significant difference in MP parameters between FVL subjects with or without a history of thrombosis.

Conclusions: This is the first study on circulating MP levels in subjects who are heterozygote for factor V Leiden. We report that circulating platelet and leukocyte MP are elevated in carriers of this mutation and may be important contributors to risk of thrombosis.

Introduction

Venous thromboembolism (VTE) and resulting pulmonary embolism are major causes of morbidity and mortality [1–3]. Resistance to activated protein C (APC) is the most common defect identified in about 20-60% of cases of spontaneous thrombosis [4]. The majority of APC resistance is attributable to the inheritance of a point mutation in the gene for Factor V (coagulation pathway zymogen) which results in a single amino acid substitution (Arg506 to Gln) known as Factor V Leiden (FVL) [5]. Case-control studies show that heterozygotes for this mutation have a 3 to 7 fold increased risk for VTE compared to healthy controls and about 10% VTE risk over their lifetime [6–8]. This risk can be further compounded by immobility, cancer as well as age and/or other genetic factors in both FVL heterozygotes and homozygotes [9–11]. Although certain plasma factors such as levels of tissue factor pathway inhibitor and endogenous thrombin potential have also been reported to be linked to the thrombotic risk in subjects with FVL, no definite risk stratification strategy using laboratory testing has yet been proven to be clinically useful for prediction of thrombosis in heterozygotes [12–14].

There has been much recent interest in the role of microparticles (MP) in the pathology of thrombosis [15,16]. These small membrane bound fragments (<1.0 μm), released from activated or dying (apoptotic) cells, are believed to be key players in the 'triad' for thrombosis [17,18]. Leukocyte MP in particular have been shown to increase thrombus production in animal models [19]. The circulating levels of MPs have been found to be higher in patients with venous as well as arterial thrombosis as compared to healthy subjects [20]. Increased platelet MP has been associated with thrombosis in platelet activation states such as heparin-induced thrombocytopaenia and acute cerebrovascular events [21–23]. Elevated endothelial MP have also been reported in the context of endothelial dysfunction in conditions such as antiphospholipid antibody syndrome, preeclampsia, renal failure and

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peripheral arterial disease as well as acute coronary syndrome [24–31]. Thus the measurement of MP has increasingly received attention as a possible diagnostic/ prognostic indicator for VTE [32]. Despite the convincing association between MP and VTE, there has been no detailed investigation into the occurrence of MP in carriers of FVL.

Circulating levels of MP may theoretically affect and contribute to the thrombogenic profile of FVL carriers. Two lines of evidence support this hypothesis: firstly, the observation that there is an approximately 10 to 20 fold reduction in the rate of APC catalysed inactivation of plasma derived factor Va when bound to synthetic phospholipid vesicles [33]. Secondly, the observation that APC can induce endothelial MP production and that APC bound to endothelial MP is no longer capable of FVa inactivation may also influence thrombosis [34]. It is possible that if circulating MP, with its phospholipid membrane protecting the FVa and inactivating the APC, are raised then it may modify the thrombogenic potential of a person. The aim of this study was therefore to evaluate circulating MP levels and procoagulant activity in a cohort of subjects who were heterozygous for FVL compared to normal individuals.

Materials and Methods

Study cohort and sample collection

The study cohort consisted of 45 heterozygous FVL subjects (13 Male, 32 Female; aged 47 ± 13 years) of which 14 had a history of thrombosis, and 45 controls (29 Male, 16 Female; aged 61 ± 15 years) with no prior history of thrombosis recruited from the Calvary Mater Hospital, Newcastle, Australia. Subjects with FVL who had thrombosis were included only if they had not received anticoagulation in the preceding 12 months. The study was carried out in compliance with the protocol and in accordance with the National Statement on Ethical Conduct in Research Involving Humans (Australia). It was reviewed and approved by the Hunter Area Research Ethics Committee and written informed consent was obtained from each participant.

Peripheral blood was collected in 0.109 M tri-sodium citrate. Platelet-free plasma was obtained by double centrifugation of whole blood for 15 min at $3000 \times g$. All samples were processed within 2 h of collection, and aliquots were stored at -80 °C until analysis. Samples were then thawed at 37°C within 15 min prior to testing.

Thrombophilia studies

Genotyping for FVL was confirmed by polymerase chain reaction using previously published methods [10] and details of the cohort have been previously reported [10,12]. All subjects and controls had normal full blood counts, PT, APTT, plasma factor levels (II, V, VII, VIII, IX, X and XI), levels of plasma antithrombin, protein C, protein S and were negative for prothrombin gene (G20210A) mutation. The entire cohort also tested negative for a lupus anticoagulant testing.

Flow Cytometry

A 50 µl aliquot of platelet-free plasma was incubated at room temperature for 15 min with combinations of CD41a-PE (Clone HIP8, BD Biosciences, CA, USA), CD62e-APC (Clone 68-5H11, BD Biosciences, CA, USA), CD45-PE-Cy5, (Clone HI30, BD Biosciences, CA, USA), and FVa-FITC (Clone V237, American diagnostics Inc, CT, USA) or appropriate isotype controls in a final volume of 100 µl of PBS. The sample was then diluted to 400 µl with filtered PBS containing 1% formaldehyde. A known number of 10 µm enumeration beads were added prior to analysis.

Data was acquired and analysed using a BD FACS Canto flow cytometer with appropriate software (BD Biosciences, CA, USA). The MP gate was established using fluorospheres of known diameter and set to record fluorescent events <1.0 µm in diameter according to a previously published method [35]. Events in the MP gate were assessed for labelling with antibody positive events to distinguish true events from background electronic noise. MP quantification was done using a modified Combes method [36]. The MP were enumerated by using the formula:

$$(10/9) \times (\text{MP count} / \text{bead count}) \times (\text{bead concentration} / \mu\text{l}) = \text{MP count} / \mu\text{l}$$

ELISA

The functional assay for the measurement of MP procoagulant activity in plasma was performed using the Zymuphen MP-Activity ELISA kit (Hyphen Biomed, Neuville-sur-oise, France) following the manufacturers instructions. Essentially, MP in the sample was allowed to bind to Annexin V on the surface of the microplate wells. Unbound particles were washed away and prothrombin added along with FXa-FVa in the presence of calcium. The production of thrombin was measured via cleavage of a chromogenic thrombin substrate producing absorbance at 405 nm and results compared to a standard curve of known MP concentration expressed in nM Phsophatidyl Serine (PS) equivalent. Thrombin generation is directly related to the phospholipid concentration in the plasma. All samples were analysed in duplicate. Only replicates that were at least 85% concordant were used in the final analysis.

Dilute Russell Viper venom test

A simplified Dilute Russell Viper Venom Test (DRVVT) was performed to assess the phospholipid concentration of the plasma samples (LA screen; Life therapeutics, French's Forest, NSW Australia). The test was performed in duplicate according to the manufacturer's instructions and results recorded on an automated coagulation analyser (SYSMEX CA-1500; Dade Behring, Newark, DE USA). This test was based on a similar principle using a modified DRVVT / Xa activation assay previously described for measuring procoagulant potential of MP [37].

Statistical analysis

All data was normalized and differences between grouped means were analysed by student's t-test. Pearson's correlation coefficients were used to assess the relationship between variables. All calculations were performed using STATA 8.0 (College Station, Texas, USA) and p-values <0.05 were considered statistically significant.

Results

Circulating MP levels are elevated in FVL

Flow cytometry was used to identify and quantify MP with specific platelet (CD41a), endothelial (CD62e) and leukocyte (CD45) surface markers (Table 1). In the overall analysis, the total number of circulating MP was significantly higher in the FVL cohort compared to the controls. Individually, all subsets of MP (platelet ,leukocyte and endothelial) were significantly elevated in FVL. The CD41a bearing MP made up the highest fraction in both the FVL and control cohorts and the proportion of MP that were also positive for Factor Va was similarly significantly higher in FVL compared to the controls. Despite the differences in the number of circulating MP between the controls and FVL cohorts, there were no differences in the prothrombinase activity recorded by the ELISA or clotting time assessed by the DRVVT with all individuals considered to be within normal range. When only the FVL cohort was considered, there was no significant difference in MP parameters between FVL patients with or without a history of thrombosis (Table 2).

Table 1
MP results for FVL and controls.

Marker	FVL (n = 45) Median(lower and upper quartile)	Controls (n = 45) Median(lower and upper quartile)	*p-value*
Total MP/ul	2100 (1527 - 3411)	1508 (1143 - 1932)	0.0021
CD45 (MP/ul)	65 (46 - 126)	37 (29 - 46)	0.0000
CD62e (MP/ul)	433 (319 - 593)	371 (287 - 417)	0.0134
CD41a (MP/ul)	1321 (894 - 2611)	945 (659 - 1393)	0.0265
Factor Va (MP/ul)	127 (98 - 166)	85 (70 - 116)	0.0004
% of Platelet MP	8 (5 - 12)	8 (5 - 13)	0.7622
ELISA (PS equivalents)	2.0 (15 - 2.7)*	2.4 (1.4 - 3.8)	0.7374
DRVVT (sec)	47 (41 - 50)	46 (41 - 50)	0.8118

*n = 30.

Functional coagulation tests do not correlate with absolute MP levels

When all of the data was included in the analysis, CD41positive MP positively correlated (R = 0.256, $p = 0.026$) and the percentage of platelet MP that expressed FVa negatively correlated with prothrombinase activity recorded by ELISA (R = -0.3085, $p = 0.007$). The relationship was true when the controls alone were sub analysed but significance was not observed in the FVL group. Neither the total number of MP or individual levels of leukocyte or endothelial derived MP correlated with the ELISA or the DRVVT results.

Discussion

This study, for the first time, demonstrates that the circulating MP, specifically platelet and leukocyte-derived, are significantly increased in subjects who are heterozygous for FVL. This reinforces the general finding of increased levels of MP seen in a variety of prothrombotic states. However, a physiological relationship between the presence of the FVL mutation and raised circulating MP levels is more difficult to discern. Individuals with FVL are known to have increased thrombin generation and exist in a prothrombotic state [12] and higher levels of MP may simply reflect the various underlying genetic/ acquired triggers for the activation of leukocytes, platelets and /or endothelium in these subjects.

The resistance to APC in carriers of FVL is at the site of attachment of APC to Factor V. Our results showed an increase in absolute numbers of FVa expressing MP in FVL subjects and this could be significant even if simply a reflection of the increased total MP (as shown by the calculation of FVa expressing CD41a positive MP in the results section). Previous in vitro studies have shown rapid inactivation of Factor Va by APC when measured in isolation but relatively high levels of thrombin generation when other coagulation / plasma factors (such as phopsholipid membrane) are present [33]. Approximately 10-20 fold reduction in APC catalyzed inactivation of Va (activated) that are bound to synthetic phospholipid vesicles has been observed [33]. Also, APC bound to endothelial MP is no longer capable of FVa inactivation [34]. Assuming that about half the circulating FV is inherently resistant to APC due to the FVL, the binding of the remainder of the FV to MP could influence the total resistance to APC. Absolute increases in circulating MP could therefore provide a protective membrane bound environment for the FV in its activated state in addition to binding APC and inactivating it. This leads to an intriguing question as to whether there could be a difference in the affinity of FVa (wild type activated FV) or FVLa (mutated activated FV) to phospholipid membrane. At this point we do not have sufficient data to allow us to comment on this possibility.

Table 2
Differences in MP parameters in FVL heterozygotes based on previous history of thrombosis*.

Marker	FVL with history of thrombosis (n=14) Median(lower and upper quartile)	FVL without history of thrombosis (n = 31) Median (lower and upper quartile)	*p*-value
Total MP/μl	1819 (1438-3149)	2137(1610 -3844)	0.23
CD45 (MP/μl)	57(39-86)	73.7(49-170)	0.14
CD62e (MP/μl)	407 (326-529)	440 (260-638)	0.69
CD41a (MP/μl)	1167 (874-2157)	1507(894-2641)	0.38
Factor Va (MP/μl)	148.7 (96-227)	122(97-166)	0.71
FVa (as % of Platelet MP)	9.5 (8.1-12.3)	7.4 (4.0-11.8)	0.12
ELISA (PS equivalents)	1.97 (1.53-2.32)[1]	2.1 (1.47-2.82)[2]	0.62
DRVVT (sec)	47.9 (44-56.4)	46.0 (40.6-50.20)	0.18

[1]n = 11, [2]n = 19.
* Only those with history of thrombosis 12 months prior to recruitment and who were not on anticoagulation for at least 6 months were included in this study.

Interestingly, there were no significant MP differences between subsets of FVL subjects with and without thrombosis. The number of heterozygous FVL subjects with thrombosis was small (n = 14) and hence some of the differences may not be apparent. Significantly increased endogenous thrombin potential has been demonstrated in FVL subjects with thrombosis compared to those without thrombosis in a previously published work [12]. Based on this it could be hypothesized that the MP in FVL subjects with thrombosis would be functionally more active than in those without thrombosis even though the actual MP counts were not significantly different. However, a significant difference was not seen in this study between MP from FVL subjects with and without thrombosis by either the functional prothrombinase assay or the modified DRVVT testing. The raised circulating MP level without apparent increase of prothrombinase activity possibly relates to the technical differences between the two approaches. There exist significant issues in standardization and detection of MP by flow cytometry as particles <0.4 μm cannot be detected by light scatter. ELISA based assays were carried out in this study to capture particles of varying size in platelet-free plasma and assess their functional activity. However, as observed in the results, there was little correlation between the functional assays and levels of MP detected by flow cytometry reflecting the ongoing technical issues in the detection and interpretation of circulating MP. One of the other draw backs of the prothrombinase assay employed in this experiment is the use of an exogenous FXa-FVa complex which binds to annexin V expressing MP on a microplate. Flow cytometry experiments suggest an increased expression, even though not reaching statistical significance, of endogenous FV on MP in FVL subjects. This increased surface expression of FV could potentially compete and interfere with exogenous FVa-FXa added to measure the prothrombinase activity of the MP surface by the ELISA method. The fact that the number of MP expressing FV by flow cytometry negatively correlated with the prothrombinase activity by ELISA supports this possibility. Experiments using controlled quantities of exogenously added FVa-FXa are being designed to investigate this finding further.

Females have been generally observed to have higher circulating levels of MP [38]. Although there were more females in the FVL group, this did skew the analysis because the mean MP levels in females, in contrast to the expectation, was actually lower than in the males from the same group (data not shown). Age could also be a confounding factor as the FVL group were significantly younger. No age specific reference ranges exists and therefore it is difficult to ascertain whether MP levels actually increase or decrease with age.

The results of this study are also somewhat restricted by some of the generic markers used to identify MP. Although the differences between the leukocyte MP in the FVL group and the controls were statistically very significant; the implications of this finding are limited by the use of only a generic CD45 marker. Microparticles of monocytic origin have been shown to be important in thrombus formation and it would be interesting to see if these were the major leukocyte subset [39]. Similarly, tissue factor bearing MP(CD142) have been implicated in numerous prothrombotic disorders and their

enumeration in the context of FVL may also be of importance [40]. Finally, the predictive value of circulating MP for thrombosis in general and FVL subjects in particular needs to be clarified by long-term prospective studies. The measurement of circulating MP employing standardized techniques in subjects at risk for thrombosis, such as those with inheritable thrombophilia, opens up the possibilities for novel predictive paradigms.

Conflict of interest statement

The authors declare that they have no conflict of interests.

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5.3 Microvesicles in pathological bleeding

Experimental data suggests that both platelet aggregation and MV generating a phospholipid surface leading to thrombus formation are equally important in maintaining normal haemostasis.[96] There is evidence that suggests that high numbers of MV, even in the setting of low platelets, may overcome platelet insufficiency and provide adequate haemostasis. Studies in patients with idiopathic thrombocytopaenic purpura who have very low levels of platelets but high MV levels do not have clinical bleeding.[97]

The ability to generate adequate MV which provides sufficient membrane surface for activated Xa and Va is the proposed mechanism for thrombus formation, even with low platelets or other consumptive coagulation factor deficiency .[98] Other mechanisms which explain how the insufficiency of platelets is overcome in certain clinical situations include platelet leukocyte interaction in models with severe thrombocytopaenia.[99] In a rare clinical context, low levels of circulating MV is associated with bleeding, as observed in Scott syndrome - a disorder with defective vesiculation of platelets. [100] We therefore hypothesized that MV have an important role to play in the pathophysiology of certain diseases associated with a bleeding phenotype. We undertook evaluation of two such clinical cohorts available at Calvary Mater Hospital where the predominant feature was either consumption of coagulation factors or low platelets. The first was a cohort of snake bite patients (consumption of coagulation factors) and the second was a group of transfusion-dependent Myelodysplasia patients (low platelets). These cohorts are described in greater detail in the sections below.

5.4 Microvesicles in snake bites

Coagulation abnormalities from snake bite envenomation in humans are commonly observed amongst all four snake families.[101] Apart from abnormal haemostasis other effects include systemic rhabdomyolysis, renal damage/failure, cardiotoxicity as well as bite site local tissue injury. Symptoms of bleeding include subcutaneous bleeds, such as bleeds from bite site, and internal bleeding ranging from mild to severe.[102] Venom induced coagulopathy (VICC) is defined as an international normalised ratio (INR) >3. Microangiopathy haemolytic anaemia (MAHA) is defined as the occurrence of thrombocytopaenia and red cell fragmentation.

Baseline tests which may be abnormal, in snake bite subjects where VICC is suspected, include whole blood clotting, PT, APTT, fibrinogen, d-dimer and platelet counts.[103] Blood film morphology is abnormal in MAHA but fragmentation is usually not prominent at presentation. Some studies suggest that use of fresh frozen plasma in those with established VICC may reduce bleeding.[104] The proposed pathology of coagulopathy and haemolysis include direct toxin induced damage of endothelium as well as venom induced activation of coagulation proteins.[105] Defibrination is also a common mechanism in some Australian snake bites leading to a bleeding phenotype.[106] A variety of other mechanisms for interference with the haemostatic pathway are also described [table 5(ii)]. The role of MV in snake bite associated coagulopathy has not been previously explored. Also, for reasons not known, MAHA occurs only in a subset of snake bite patients. Therefore, a cohort from the Australian Snake bite Project (ASP) was studied for the role of MV and its association with MAHA in the context of snake envenomation. We specifically evaluated MV levels using flow cytometry in cohort of snake bite patients with a focus on the relationship of MV to snake bite patients having VICC without MAHA and VICC with MAHA.

Table 5(ii). Effects of snake venom on haemostasis, adapted from references[105,107,108]

Toxin	**Effect**
Procoagulants	Factor V activating Factor X activating Factor IX activating Prothrombin activating Fibrinogen activating
Anticoagulant	Protein C activating Factor IX/X inactivating Thrombin inhibitor Phospholipase A_2
Fibrinolytic	Fibrin(ogen) degradation Plasminogen activation
Vessel wall interactive	Haemorrhagins
Platelet activity	Platelet aggregation inducers Platelet aggregation inhibitors
Plasma protein activators	SERPIN inhibitors
Others	Microvesicles of different cellular origin

Aim 4:

To evaluate of levels of circulating MV in a cohort of snake bite patients with venom induced consumptive coagulopathy and microangiopathic hemolyic anaemia.

PAPER 6: Enjeti AK, Lincz LF, Seldon M, Isbister GK. Circulating microvesicles in snakebite patients with microangiopathy. Res Pract Thromb Haemost 2019;3:121-5.

Key learning and reflections

Red cell MV are elevated in patients with VICC and MAHA. Endothelial MV were decreased in snake bite patients but the mechanism needs further investigation.

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BRIEF REPORT

Circulating microvesicles in snakebite patients with microangiopathy

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Abstract

Background: Venom-induced consumption coagulopathy is a common consequence of snake envenoming that can lead to life-threatening hemorrhage, and is associated with microangiopathic hemolytic anemia (MAHA), acute kidney injury and thrombocytopenia. The role of microvesicles (MV) in snakebite patients has not been previously investigated.

Objective: To compare changes in subsets of circulating MV levels in snakebite patients with venom induced consumption coagulopathy and with or without microangiopathic hemolysis to those of healthy controls.

Methods: This study used samples from patients recruited to the Australian Snakebite Project (ASP) with snake envenoming, including bites by brown snakes, tiger snakes, and taipans. Citrated blood from envenomed patients was collected, processed, and stored according to a national standardized protocol. Full blood count and coagulation parameters were measured as per routine clinical care and blood films were examined for evidence of hemolysis. Baseline coagulation parameters were measured on a Behring Coagulation System. Flow cytometry was performed to detect CD41a (platelet), CD62e (endothelial), and glycophorin (red cell) MV. The results were analyzed using BD software and appropriate statistical tools.

Results and Conclusions: The red cell MV in snakebite patients with MAHA (n = 13) were significantly higher than those without MAHA (n = 17) while there was no significant difference in platelet MV levels between the snakebite patients with and without MAHA. Interestingly, the endothelial MV were reduced in all snakebite patient samples compared to the control samples. Measuring red cell MV at presentation could be useful as a predictive marker for MAHA in patients with snakebites.

KEYWORDS
coagulopathy, extracellular vesicles, microangiopathy, microvesicles, snakebite, venom

Essentials

- Snake bites are associated with venom induced coagulopathy, microangiopathic hemolytic anaemia (MAHA) and thrombocytopaenia.
- The mechanisms of MAHA and role of MV in its pathophysiology in snake bite are not known.
- This results of this study show that red cell MV are increased in snake bites associated with MAHA whilst endothelial MV are reduced in all snakebites.
- Evaluation of circulating endovascular microvesicles provide an important mechanistic tool and have a potential role as predictive markers for studying the effects of snake venom.

1 | INTRODUCTION

Snake venoms are a complex mixture of proteins and polypeptides which cause a broad range of toxic effects when released by the snake into the prey during a bite. Although snake toxins have probably evolved to assist in prey capture and defense, they inadvertently cause a range of systemic effects when humans are bitten. Many snake venoms contain procoagulant toxins that activate the clotting pathway, which results in venom induced consumption coagulopathy (VICC) in human envenoming, and this is the most common important clinical manifestation of snakebites worldwide.[1,2] Australian elapids contain serine proteases, which closely resemble the mammalian prothrombinase complex.[3]

Exposure to these prothrombin activators leads to widespread activation of the coagulation pathway resulting in consumption of the key clotting factors, including fibrinogen, FV, and FVIII.[2,4] More recently, microangiopathic hemolytic anemia (MAHA), acute kidney injury, and thrombocytopenia have also been recognized to be associated with VICC. Although this is thought to be a form of thrombotic microangiopathy, the pathophysiology remains unclear.[2-4]

Circulating microvesicles (MV) or small cell-derived extracellular vesicles have been implicated in hemostatic disorders—an increase in MV associated with pathological thrombosis, and a decrease contributing to potential bleeding.[5-7] Several cell-derived MV are frequently found in the circulation—these include red cell, platelet, leukocyte, and endothelial cell-derived.[8,9] Flow cytometry is a popular and easily accessible technique for measuring MV and determining their cellular origin.[10]

The role of circulating MV in snakebite patients is not known and we present the results of a unique investigation of MV in snakebite related VICC and MAHA. Changes in MV levels after envenoming could indicate direct toxin mediated damage to the endovascular system which has been hitherto difficult to evaluate or measure in a systematic fashion. To explore MV in snake envenoming further, we analyzed the levels of circulating platelet, endothelial, and red blood cell-derived MV in patients recruited to the Australian Snakebite Project (ASP) with confirmed envenoming from brown snake, tiger snake, or taipan.

2 | METHODS

Samples and clinical information were sourced from patients recruited to the ASP with elapid envenoming (including brown snake, tiger snake, and taipan envenoming), as previously described.[11] Citrated plasma was collected from each snakebite patient recruited to the study. Peripheral blood was collected in 0.109 mol/L tri-sodium citrate and platelet-free plasma was prepared by double centrifugation of whole blood for 15 minutes at 2500×*g*. All samples were processed within 2 hours of collection, and aliquots were stored at −80°C for further batched analysis. Samples were then thawed at 37°C, 15 minutes before testing. Control samples were obtained from healthy volunteer blood donors after appropriate consent—these were processed and stored in an identical fashion to the patient samples.

Prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, and D-dimer concentrations were measured on a Behring Coagulation System (BCS; Dade Behring, Marburg, Germany) at a centralized laboratory. Full blood count was measured as per local routine laboratory protocol and blood films were examined by an independent pathologist for presence of red cell fragmentation for confirmation of MAHA. VICC was defined as an international normalized ratio (INR) >3 with undetectable fibrinogen, and MAHA as the occurrence of thrombocytopenia and red cell fragmentation.[11]

Flow cytometry was done on a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) with combinations of antibodies specific for platelet marker (CD41a-PE; Clone HIP8, BD Biosciences), red cell marker (CD235a-APC; Clone GA-R2, BD Biosciences), endothelial cell marker (CD62e-APC; Clone 68-5H11) and appropriate isotype controls in a final volume of 100 μL of PBS. Megamix beads (Biocytex, Marseille, France) were used to gate events less than 1 μm in diameter and analysis was undertaken using FACSDiva software. Control samples were obtained from healthy volunteer blood donors from the Australian Red Cross blood service. They ranged in age from 21 to 60 years and had a mix of both sexes. These subjects were screened for any significant medical conditions and samples obtained were processed within 4 hours from collection in a citrated tube in a manner similar to the snakebite patients' samples.

Microsoft Excel 2007 and SPSS, (IBM SPSS Statistics for Macintosh, Version 19.0., IBM Corp., Armonk, NY, USA) were used to collate and evaluate the data. Prism 7.0a (Graphpad Software, La Jolla, CA, USA) was used for graphical representations and nonparametric analysis was undertaken to evaluate differences given the large standard deviations in the MV levels. The normal cohort, VICC without MAHA (VICC no MAHA) and VICC with MAHA

TABLE 1 Comparison of levels of microvesicles (MV) in healthy controls and snakebite patients with VICC (no MAHA) and with VICC + MAHA

Samples/variable	Healthy controls (n = 28)	VICC (no MAHA) (n = 17)	VICC + MAHA (n = 13)
Age	30 ± 14	38 ± 9.8	38 ± 19.5
Gender (male, %)	54	76	77
Hb (g/L)	144 ± 9	136 ± 15	85 ± 16
Platelet count (10^9/L)	265 ± 85	141 ± 75	35 ± 13
Platelet MV/μL	1073 (614-1630)	1509 (1055-2387)	1147 (541-5168)
Endothelial MV/μL	359 (289-431)	<50	<50
Red cell MV/μL	<50	78 (7-180)	626 (393-1019)

Data presented as mean ± SD for parametric distribution (age, Hb and platelet counts) and median (interquartile range) for non-parametric distribution (MV levels).

(VICC + MAHA) groups were compared. One way ANOVA (Kruskal-Wallis) test was used for non-parametric comparisons between the groups and a level of 0.05 was set for statistical significance. The study was approved by the local ethics committee (Hunter New England ethics committee approval number 06/12/13/5.05) and by all major State and Territory human research ethics committees for the hospitals involved in the ASP.

3 | RESULTS AND DISCUSSION

A cohort of snakebite patient samples who had envenoming and VICC, and had baseline samples collected on admission to hospital, were evaluated. There were 17 patient-samples with VICC alone (no evidence of MAHA; designated as VICC no MAHA) and 13 patient-samples with VICC with MAHA (designated as VICC + MAHA); and 28 normal healthy controls. The age range for all snakebite patients was 24-73 years and there were more males than females (see Table 1). However, the differences between the age and gender between the groups compared was not statistically significant (Kruskal-Wallis test for age and *z*-test for gender). All envenomation was by brown snake bites apart from five patients (two tiger snake and three where the type could not be determined due to insufficient pre-antivenom samples). results for baseline blood counts and circulating MV levels in the three groups are shown in the table and illustrated in Figure 1. As expected, snakebite patients with MAHA had lower hemoglobin and platelets compared to controls and those without MAHA ($P < 0.001$). Platelet MV were significantly elevated in snakebite patients without MAHA (VICC no MAHA) compared to controls ($P = 0.009$), but there were no differences between the snakebite patients with and without MAHA ($P = 0.21$). Interestingly, the endothelial MV were significantly reduced in all snakebite samples compared to the controls ($P < 0.001$). The levels of red cell MV in snakebite subjects with MAHA (VICC + MAHA group), however, were significantly higher than those without MAHA ($P = 0.002$) and when compared to controls (see Figure 1).

A markedly elevated red cell MV level may be used as a surrogate marker for MAHA whereas thrombocytopenia alone may

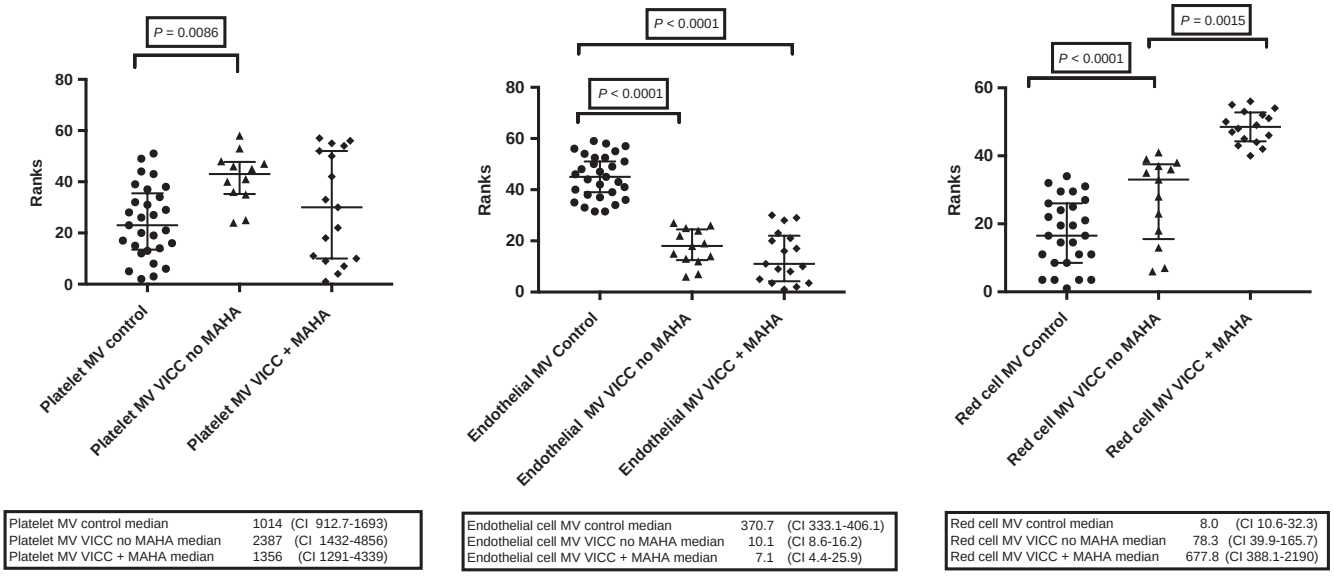

FIGURE 1 Median and 95% confidence interval for platelet, endothelial, and red cell microvesicles between the snakebite patients with VICC (no MAHA) and with VICC + MAHA. The significant *P* values are shown for differences between the groups for one way ANOVA (Kruskal-Wallis test)

not be able to discriminate between those snakebite patients with evolving MAHA and those without. The correlation between INR, PT, and aPTT and MV levels could not be quantified because most subjects had un-recordable parameters due to VICC, and there was likely to be no difference in these parameters between the two groups.

Increased levels of red cell MV in snakebite patient samples with MAHA is consistent with red cell breakdown and hemolysis. If this is a consistent finding then measuring MV at presentation could be useful as a predictive marker for MAHA in snakebite patients. This suggests an alternative mechanism for the observed microangiopathy other than endothelial damage alone due to the snake toxin. The rapid generation of red cell MV may subsequently lead on to a consumptive coagulopathy and microvascular thrombosis, which is often accompanied by thrombocytopenia that is typical of MAHA. It is also possible that some of the "vesicles" may represent red cell fragments rather than "microvesicles" and it may be considered technically challenging to separate the two populations by flow cytometry alone. However, majority of these fragments as seen on a blood film are usually in the size range of 1 μm or above, and in the range of the size of large platelets.[12] In previous studies evaluating the detection of fragmented red cells, using an automated hematology analyzer, have also used the principle of light scatter. The average dimension of the fragments was about 30 fL with a unidimensional size of a few microns when compared to a red cell which ranges between 80 to 100 fL with a diameter of about 8 μm.[13,14] The gating strategy used in our experiments excluded events larger than 1 μm in the MV gate therefore ensuring only red cell MV were enumerated.

The reason for apparent low levels of circulating endothelial MV could be due to changes in the size or surface antigen expression of the MV which may be altered in response to envenomation, resulting in MV that are undetectable via our method of flow cytometry. This was an unexpected finding that requires further investigation.

Limitations of this study include that we did not perform any functional studies in MV isolates or use alternative methods to evaluate MV apart from flow cytometry. Inter-laboratory as well as inter-instrument variability in flow cytometry is a concern despite the fact that it is an easily and widely available technique. Also, MV smaller than 200 nm are usually not detectable by flow cytometry. The use of isotype controls, correlation with functional testing and participation in global standardization initiatives may reduce this variability.[15] Given the small sample size, we did not stratify the results according to gender or comorbidity status but this may be important to explore in a larger cohort.

There may be some variability in how snake venom interacts with the human coagulation system. However, all of the snake venoms in the current study are well known to contain procoagulant toxins that closely resemble part or all of the mammalian prothrombinase complex and produce identical clinical sequelae. The toxin in brown snake is the most potent, being most similar to the FXa-Va complex, and able to directly cleave prothrombin to produce activated thrombin. The procoagulant toxin in tiger snake resembles FXa and requires completion of the complex from human plasma FVa to enable thrombin activation.[1]

In summary, we provide the first report of changes in endovascular MV in snakebite patients and their possible role in the pathophysiology of snakebite-related MAHA. This study provides proof of principle that MV levels could be a marker for direct toxin mediated damage to red cells, platelets, and endothelial cells, that has been hitherto difficult to define. We established that flow cytometric assessment of MV could be used as an important technique for measurement of direct toxin mediated damage to red cells in snakebite associated MAHA.

RELATIONSHIP DISCLOSURES

AKE is a recipient of NSW Health Pathology/HCRA/CMN translational research fellowship. This study was partly supported by Calvary Mater Research funds and NSW Health Pathology–Hunter Trust Funds. GKI is supported by a NHMRC Senior Research Fellowship ID1061041 and the Australian Snakebite Project is partially funded by an NHMRC Centre for Research Excellence Grant ID1110343. We acknowledge the various hospitals who participated in ASP study and provided the samples as well as the study pathologist who provided review of blood films. There are no conflicts of interest for any of the authors.

AUTHOR CONTRIBUTIONS

AKE, LL, MS, and GI conceived the project; GI provided access to samples and interpretation of data; MS provided access to coagulation testing and flow cytometry; AKE carried out the project and data analysis; AKE, LL, MS, and GI were responsible for data interpretation and manuscript.

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5.5 Microvesicles in haemopoietic dysregulation

5.5.1 Microvesicles in clonal stem cell disorders – Myelodysplasia

Myelodysplasia (MDS) is a group of clonal bone marrow stem cell disorder with abnormal haematopoiesis resulting in low blood counts and transfusion dependence.[109] MDS is characterised by leucopaenia, anaemia and thrombocytopaenia. MDS increases the risk for transformation to acute leukemia. MDS is characterised by a range of microscopic features which show disordered maturation of marrow precursor cells. The most specific features are usually noted in the megakaryocyte or platelet precursor lineage [see fig 5(b)] MDS is a genetically heterogeneous disorder and scoring systems have been developed to assess risk for transformation. These risk tools are based on transfusion dependence, blood counts, blast counts and cytogenetic abnormalities in the bone marrow at diagnosis. These risk scores have been studied prospectively, published by WHO and an international study group, in large number of patients with Myelodysplasia indicating higher scores relate to poorer prognosis.[96] However, the risk scores do not always predict the transfusion dependence or bleeding tendency accurately and patients who have similar scores have vastly different clinical phenotypes.

Circulating MV level and function in Myelodysplasia has not been previously studied as there are no published articles in this area. Given the interaction of the MV with the endothelium, it is possible that MV are involved in endothelial integrity and disease characteristics such as transfusion dependence in MDS. One could postulate that there may be ways in which MV interact with endothelium or other normal bone marrow cells to cause differential transfusion dependence. The lack of procoagulant P-selectin may be resulting in reduced haemostatic potential.[110] The endothelial integrity and angiogenesis may also potentially be affected by abnormal circulating MV.[111] Another potential mechanism would be the endoglin (CD105)

bearing MV and their potential impact on both endothelial cells as well as stem cells. In previous studies, increased levels of soluble CD105 have been associated with myeloid malignancies. [110,112] It is possible that a proportion of what has been identified as soluble CD105 may in fact be expressed on MV. It was therefore important to evaluate the role of circulating MV, particularly those from platelet and endothelial cells, in a cohort of MDS subjects and its association with disease characteristics.

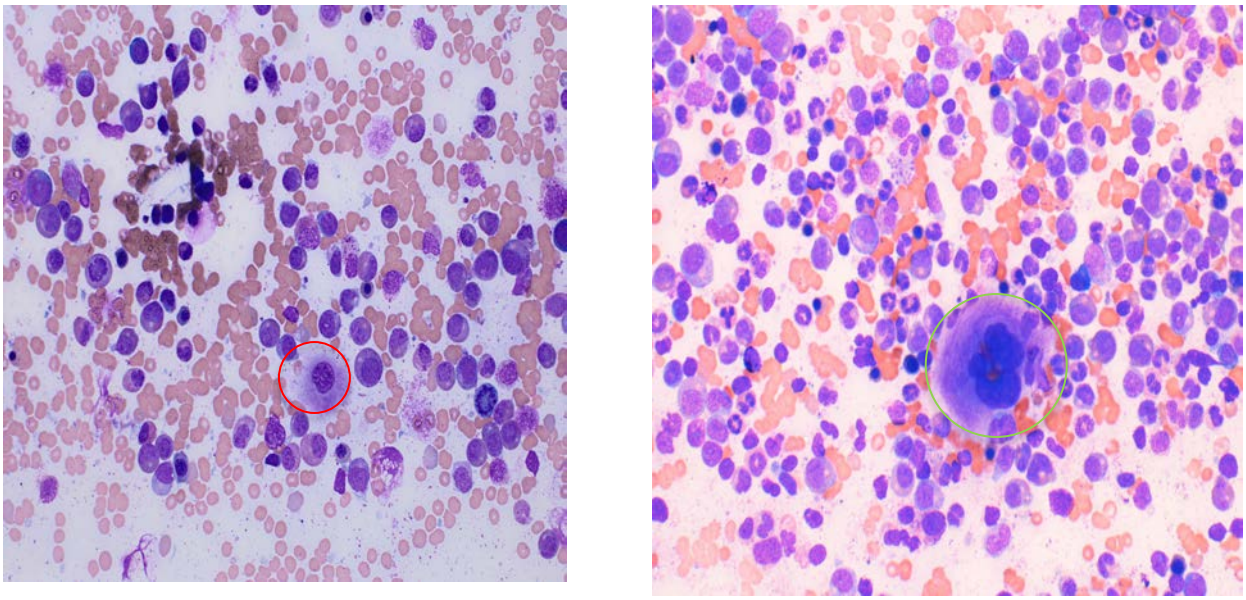

Figure 5(b). Microscopic image of (panel left) bone marrow aspirate in MDS stained with geimsa with disordered haemopoietic precursor cells and a characteristic micromegakaryocyte, abnormal platelet precursor (red circle) at 20x magnification; (panel right) a normal megakaryocyte, a normal platelet precursor, is shown on the right for comparison (green circle) this is much larger cell, several times larger than adjoining myeloid precursors and have multi-lobulated nuclei at 20x magnification. Courtesy Haematology Unit, Calvary Mater Newcastle.

5.5.2 Microvesicles and miRNA

The microRNA (miRNA) are a group of small (19-25 nucleotides) ribonucleotides now known to be directly involved in regulating gene expression and have been implicated as major contributors to the pathogenesis of certain endovascular disease states such as diabetes and stroke.[5] A variety of functions have been ascribed to miRNA and in particular, the way in which they interact with the endothelium is diverse.[39,113] This connection is now being extensively investigated with pro- and anti-angiogeneic activities of various miRNA. Intriguing evidence for a role of miRNA in paracrine cell regulation comes from the recent finding that miRNA can be transferred between cells via MV.[36,39]

5.5.3 MV miRNA in endothelial integrity

The recent discovery of miRNA contained in circulating cell derived microvesicles may provide some insight into some of the complexities of maintaining endothelial integrity.[113] Endothelial cells line the blood vessels and confine the cellular components of blood within the walls. The loss of the endothelial lining leads to leaking of red cells, leucocytes or platelets across the vessel wall leading to bleeding and inflammation. In the presence of adequate numbers of platelets, the leak would be sealed by platelet and coagulation interplay resulting in haemostasis.

Many of the circulatory proteins involved in coagulation such as tissue factor and P-selectin are also known to influence angiogenesis.[114] Thus, both platelet derived and tissue factor bearing MV may promote angiogenesis. A recent study showed that MV isolated from diabetic patients with vascular complications disrupted endothelial tube formation *in vitro*.[115] It is known that platelets play an important role in endothelial integrity but the mechanisms through which this is affected is as yet unclear. Platelets are thought to support the resting vascular endothelium by several mechanisms and platelet components (such as MV) may

promote the growth of endothelial cells.[111] It is hypothesized that MV play an important role in endothelial integrity and potentially could be the pathway by which platelets interact with endothelium but this remains to be completely elucidated.[29] [116] One could postulate that there may be ways in which MV interact with endothelium including via direct miRNA transfer to influence endothelial integrity.

The role of miRNA in endothelial cell function, regulation and proliferation has been widely explored.[117] The miRNA with published lines of support in experimental models include miR-126, miR-10a and the Let-7 cluster. The miRNA may have diverse, sometimes opposite roles such the pro-angiogenic miR-17-92 cluster and the anti-angiogenic miR-221 and miR-222.[118] The regulation and /or dysregulation of angiogenesis or endothelial function by miRNA during thrombosis, atherosclerosis, cancer and tumour progression has also been described.

5.5.4 miRNA in haemostasis and haemopoiesis

The differential expression of miRNA to evaluate their potential role particularly with respect to venous thromboembolism has also been explored. A recent meta-analysis of the literature identified 13 differentially expressed miRNA, with 8 upregulated and 5 downregulated miRNA species.[119] The miRNA that regulate PI3K (phosphoinositide 3-kinases) signalling pathway have been implicated in regulating endothelial apoptosis and therefore risk of thrombosis. The VEGF (vascular endothelial growth factor) pathway is another mechanism by which miRNA can influence angiogenesis.[120] Both these pathways converge at the crossroads of angiogenic signalling, cell proliferation, survival and metabolism – key signals for both physiological and pathophysiological roles.

Haemopoiesis is a multistep complex hierarchical process where specialized blood cells are produced from pluripotent stem cells. A 100 billion blood cells are produced in the human

body daily the regulation of which involves several levels of control.[121] In addition to signalling events, transcription and epigenetic changes, the role of miRNA in this differentiation process is being increasingly understood. Previous studies show that miR-130a, miR-199 are upregulated in whereas others such as miR-145 and miR-146 are downregulated in the bone marrow of MDS patients [figure 5(c)]. [121]

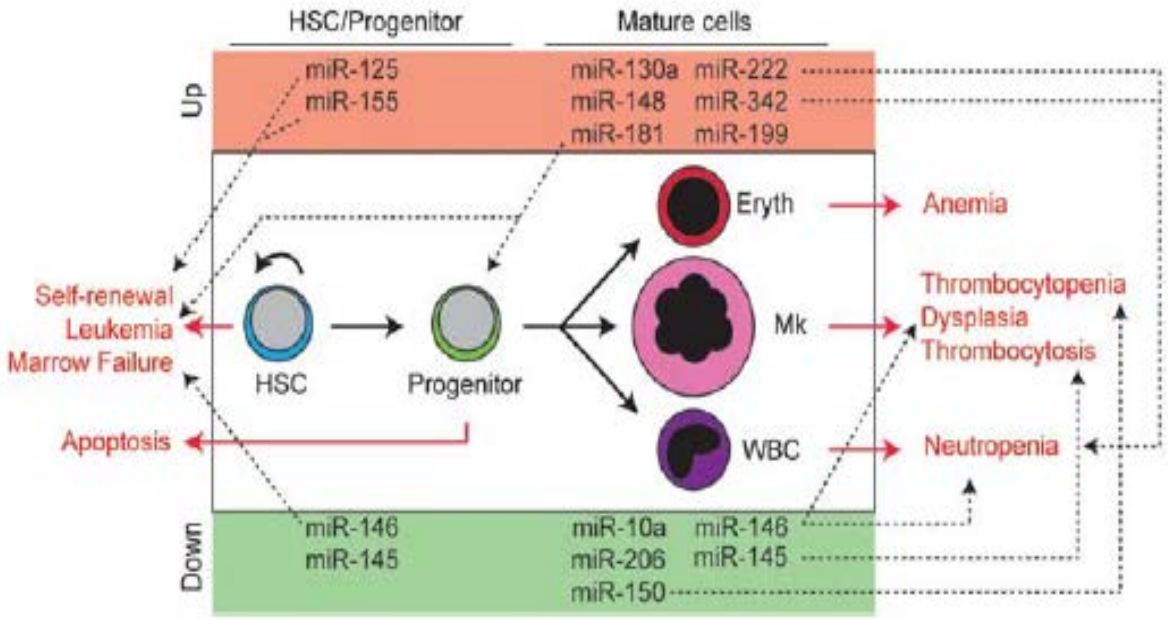

Figure 5(c). Haemopoietic stem cells give rise to myeloid precursors and their differentiation is influenced by various miRNA levels, reproduced from reference.[121]

It is known that dysregulated miRNA can negatively impact haemopoiesis. In an ex-vivo acute leukemia model, MV derived from myeloid leukemia were shown to influence normal haemopoietic stem cell survival via transfer of specific miRNA.[122] The MV from the abnormal clone can therefore potentially affect the remaining normal bone marrow cells to impair normal haematopoiesis.[5]

We hypothesized that the MV levels may be increased in MDS subjects and their profile will be different from normal healthy subjects. We also postulated that MV levels may be associated with MDS disease characteristics as well as risk scores. We wished to explore

the small RNA cargo in MV isolated from MDS subjects, with a focus on miRNA, given their potential role in endothelial integrity, haemostasis and haemopoiesis.

Aim 5:

. To analyse the levels of MV in a cohort of myelodysplasia subjects by

a) both numerical and functional tests and correlate with disease risk scores, blood counts and

b) to isolate and characterise the small RNA and specifically, miRNA profile of circulating MV in myelodysplasia patients

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Key learning and reflections

MV levels detected by flow cytometry are generally decreased in MDS compared to normal healthy controls. MV in MDS have significantly lower procoagulant function by both ETP and XaCT assays. However, no differences were observed when MV levels were measured by NTA. Certain miRNA species were differentially expressed in MDS.

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Circulating microvesicles are less procoagulant and carry different miRNA cargo in myelodysplasia☆

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ABSTRACT

Background and aims: Myelodysplasia (MDS) is characterised by abnormal haematopoiesis and increased risk of bleeding. Microvesicles (MV) play a key role in coagulation and their impact in MDS is unknown.
Methods: Platelet free plasma from 35 red-cell transfusion-dependent MDS patients and 15 controls were analysed. Pro-coagulant function was assessed by the XaCT assay and by thrombin generation (ETP). Total MV were enumerated by nano-tracking analysis. MV subsets were quantified by flow cytometry after staining with specific antibodies for various endovascular cell types. Small RNA was quantitated and sequenced. The MV measurements were correlated with MDS clinical risk scores and level of transfusion dependence.
Results: The pro-coagulant function of MV was significantly lower in MDS. All the MV subtypes, as measured by flow cytometric markers, were also significantly lower. The small RNA and miRNA cargo were significantly higher in MDS. The miRNA profile showed that mir-28 and mir-LETD7 were under expressed whilst mir-584J and mir-4485 were over expressed in MV from MDS.
Conclusions: Circulating MV in MDS show reduced pro-coagulant functional activity, reduced subtypes by flow cytometry and significantly different miRNA content. However, the levels or subtypes of MV did not predict the clinical phenotype or level of transfusion dependence.

1. Introduction

Myelodysplasia (MDS) is a bone marrow dysfunction characterised by disturbed haematopoiesis. Although there are increased numbers of bone marrow precursors in most patients, abnormal haemopoietic maturation leads to fewer red, white blood cells and platelets [1]. Along with clinical features of anaemia, neutropaenia, and thrombocytopaenia, a subset of patients with MDS will experience serious bleeding, and this may not necessarily be dependent on platelet counts [2,3].

Experimental data suggests that both platelet aggregation and phospholipid surface to facilitate thrombus formation are equally important in maintaining normal haemostasis [4]. There is evidence to suggest that high numbers of circulating microvesicles, even in the setting of low platelets, may overcome platelet insufficiency and provide adequate haemostasis. For example, studies show that patients with idiopathic thrombocytopaenic purpura who have very low levels of platelets along with high MV levels do not have clinical bleeding [5]. The ability to generate adequate MV in this instance provides sufficient membrane surface for activated coagulating factors Xa and Va to produce thrombin and generate a fibrin plug in spite of low platelets [6]. Other mechanisms to explain how the insufficiency of platelets is overcome include platelet interaction with leukocyte-derived MV, in models of severe thrombocytopaenia [7]. In a rare clinical context, low levels of circulating MV is associated with bleeding, as observed in Scott syndrome - a disorder with defective vesiculation of platelets [8].

Circulating MV also carry a cargo of small and miRNA which has potential to influence the bone marrow microenvironment as well as the endovascular compartment [9]. Free miRNA in circulation

☆ No conflicts of interest for any of the authors.

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complexes with many RNA binding proteins, which regulate its integration in tissue. However, miRNA in MV is unbound and readily available to regulate nuclear transcription and influence cell function [10]. MDS is often characterised by marked increase in apoptosis and resultant vesicular debris/bodies in the marrow generating abnormal levels of MV [11]. We therefore hypothesized that both levels and functional attributes of circulating MV in MDS, as well as their small/miRNA cargo, contribute to the pathophysiology of the disease. Hence the aim of the study was to examine these aspects of MV from MDS compared to normal healthy subjects.

2. Methods

After informed consent, samples were collected from 35 MDS and 15 age similar healthy subjects. Ethics approval was obtained from Hunter New England local health district research ethics committee (06/12/13/5.05).

The healthy controls were voluntary blood donors and these subjects were screened for any significant medical conditions. The control samples were collected in a citrated tube and processed in a manner similar to the MDS cohort.

2.1. Sample preparation

Peripheral blood was collected in 0.109 M tri-sodium citrate and platelet-free plasma was prepared by double centrifugation of whole blood for 15 min at 2500 $\times g$ at room temperature. All samples were processed within 2 h of collection, and aliquots were stored at −80 °C for further batched analysis. Samples were then thawed at 37 °C, 15 min before testing. In the transfusion dependent MDS cohort, blood samples were collected at the nadir of their blood counts i.e. just before the next transfusion.

2.2. Functional coagulation based studies

The factor Xa activation test (XaCT) was performed using the commercially available XaCT test kit (Haematex, Australia) [12]. The measurement of procoagulant activity of MV is based on the ability of vesicles expressing phosphatidylserine to generate Xa. It was performed in duplicate on an automated coagulation analyser (SYSMEX CA-1500; Dade Behring, Newark, DE USA) and results read from a standard curve generated by dilution of a plasma calibrator (expressed in ng/ml).

2.3. Calibrated Automated Thrombogram measurements

Thrombin generation experiments were performed at 37 °C on a fluorometer (Fluoroskan Ascent, Thermo Electron Corporation, Vantaa, Finland) and analysed using the Calibrated Automated Thrombogram (CAT). A sample volume of 80 µl was incubated with 20 µl calibrator or 20 µl specific MP-reagent (Thrombinoscope, Maastricht, The Netherlands) which has been reported to be sensitive to tissue factor bearing MV [13]. Thrombin generation was measured in real time and analysed by the Thrombinoscope software version 3.0029 (Thrombinoscope, Stago Group, Maastricht, The Netherlands) for the endogenous thrombin potential (ETP).

2.4. Nanoparticle tracking analysis (NTA)

The MV enumeration by nanotracking was undertaken on a Nanosight NS500 instrument (Malvern instruments, Malvern, United Kingdom). The scatter and the fluorescence measurements were recorded after incubation with Qdot 625 stain (Life Technologies/Thermo Fisher Scientific, MA, USA) in a dilution of 1:100 with PBS. The capture settings such as camera, focus and gain, were optimized so that particle tracks were clearly visible. If the capture was suboptimal (i.e. event capture rate either < 20 or > 200 tracks) further dilutions were undertaken so that at least 100 completed tracks were recorded [14]. Measurements were taken in triplicate, in scatter and fluorescent mode, for analysis using the Nanosight software (version 2.3 and 3.1); the captured video data was analysed for scatter events (total) and the fluorescent events (total).

2.5. Flow cytometry

Antibodies used included platelet marker CD41a-PE (Clone HIP8, BD Biosciences, CA, USA), red cell marker CD235a-APC (Clone GA-R2, BD Biosciences, CA, USA), endothelial marker CD105-PE (Clone IG2, Beckman Coulter, Marseille Cedex, France), CD14-PC (Clone RMO52, Immunotech, Marseille, France) and tissue factor TF-FITC (Clone VD8, American diagnostics Inc., CT, USA). A 10 µl aliquot of platelet-free plasma in a final volume of 100 µl of PBS (phosphate buffered saline) was taken and incubated (in the dark) with appropriate antibody or isotype control at room temperature for 15 min. For experiments with annexin V-APC (eBioscience, CA, USA), used for marking phosphatidylserine (PS), this incubation was done in a total 50 µl of binding buffer. Then the sample was diluted to 400 µl with filtered PBS or 450 µl of calcium rich buffer respectively. Prior to the analysis, a predetermined number of 10 µm enumeration beads (CountBright beads, Molecular Probes, Life Technologies, Oregon, USA) were added. The flow cytometer was standardized as per the 'ISTH workshop for standardization of flow cytometry for Microparticles' [15]. The gating was performed using Megamix beads (Biocytex, Marseille, France) on a BD FACS Canto instrument (BD Biosciences, San Jose, California, USA) A total of 35 MDS and 11 control samples were analysed, after exclusion of 4 control samples with poor staining. The analysis was undertaken on FACSDiva software.

2.6. Small RNA quantitation

MV were pelleted by centrifuging 1.2 ml of plasma at 21,000 $\times g$ for 60 min at 4 °C. Then small RNA was extracted using the Norgen kit (Norgen Biotek, ON, Canada) for RNA extraction using a slurry based method according to the manufacturer's instructions. The 100 µl of RNA containing eluate was reduced to a final volume of about 7 µl with a speedy-vac. For each sample, 1 µl of the concentrated RNA eluate was used for measurement of small RNA concentration by Agilent Bioanalyzer Small RNA Assay using Bioanalyzer 2100 Expert instrument (Agilent Technologies, Santa Clara, CA).

2.7. miRNA profiling

2.7.1. NGS Library generation and sequencing

Small RNA libraries were constructed with the CleanTag Small RNA Library Preparation Kit (TriLink, Cat# L-3206) according to the manufacturer's protocol. The final purified library was quantified with High Sensitivity DNA Reagents (Agilent Technologies, PO# G2933-85004) and High Sensitivity DNA Chips (Agilent Technologies, PO# 5067-4626). The libraries were pooled, and the 140 bp to 300 bp region was size selected on an 8% TBE gel (Invitrogen by Life Technologies, Ref# EC6215). The size selected library was quantified with High Sensitivity DNA 1000 Screen Tape (Agilent Technologies, PO # 5067-5584), High Sensitivity D1000 reagents (Agilent Technologies, PO# 5067-5585), and the Tailor Mix HT1 qPCR assay (SeqMatic, Cat# TM-505), followed by a NextSeq High Output single-end sequencing run at SR75 using NextSeq 500/550 High Output v2 kit (Cat #FC-404-2005, Illumina, San Diego, CA) according to the manufacturer's instructions.

2.7.2. Bioinformatic analysis

Data consists of single end 76 bp long reads, on 30 samples (10 normal/controls and 20 MDS patients in whom sufficient plasma aliquots were available) for small RNA isolated from MV fraction within the plasma samples. Based on the small RNA species composition,

quality control data, hierarchical clustering based on raw counts, four samples were excluded from the control group due to the higher than 75% proportion of unknown/unmapped reads to identified features. A further six samples from the MDS group were also excluded due to high unknown proportion/unmapped reads. The final cohort for miRNA expression analysis comprised of 6 normal and 14 MDS samples. Data was contig-quantised into regions longer than 20 bp with a maximum gap of 20 bp and at least two reads using Seqmonk v1.41. 85% of contigs had length < 116 bp, and 13.5% of length between 116 bp and 208 bp. Annotation to known features was performed on the significant results.

2.8. Statistical considerations

The statistical analysis was performed using Prism 7 (GraphPad, OK USA). The variables in the data set were evaluated for normality of distribution by a normality test such as the Kolmogorov-Smirnov test to decide whether a nonparametric rank-based analysis or a parametric analysis should be used. The differences between any two groups was assessed by the Mann-Whitney *U* test, and between repeat measures by ANOVA with a p-value < 0.05 considered as statistically significant.

Differential expression analysis was performed in the R statistical environment on contig features using the edgeR and DESeq2 packages, contig counts normalised to library size. Consensus between methods was higher than 70% at the 0.05 FDR level. Significant contig features were then annotated to transcripts from the ENSEMBL GRCh38v90 reference. Two analysis algorithms were used - Bowtie2 and BLAST with parameters for small RNA alignment. Results were considered significant only in those sequences which showed significance across the two algorithms. The significance was set at a p value of 0.05 using adjusted p values for an optimized false discovery rate approach.

3. Results

3.1. Patient characteristics (MDS)

There were 35 MDS subjects, all red cell transfusion-dependent, who were recruited prospectively into the study. The demographic data for the normal and MDS cohorts is shown in Table 1 - the bone marrow characteristics and IPSS risk scores were obtained at diagnosis. The time from diagnosis ranged from < 6 months to 5 years with majority of diagnosis within 2 years (65%, 23/35). The age is recorded at the time of the MV sample collection. The mean age of MDS patients was 78 years (range 56–91) which was significantly higher than the mean age of the normal subjects was 65 years (range 60–73). The full complement of clinical details, including full blood count parameters and WHO bleeding score, is also provided (see Supplementary Table 1).

Table 1
Baseline demographics for normal and MDS. The risk categories for MDS are also detailed. Results presented as median and range for all continuous variables.

	Normal (n = 15)	Range	MDS (n = 35)	Range	p value
Age years	64	60–73	78	56–91	< 0.0001
Gender (M, %)	47		75		0.0049
Hb g/l	127	116–145	84	59–120	< 0.0001
White cell counts 10^9/l	5.4	4.3–8.6	4	1–18	0.1315
Platelets 10^9/l	201	111–313	79	4–434	0.0043
IPSS (n, %) n = 28					
Low			10 (36)		
Intermediate 1			11 (40)		
Intermediate 2			5 (17)		
High			2 (7)		
IPSS-R (n, %)					
Very low			3 (10)		
Low			10 (35)		
Intermediate			9 (43)		
High			4 (15)		
Very high			2 (7)		

3.2. MV in MDS - functional studies

The procoagulant function by ETP and XaCT were both significantly lower in MDS (p < 0.0001 for both) as shown in Fig. 1. The median ETP in MDS was 1094 RFU (relative fluorescence units)/min (IQR of 933–1212) which was nearly half that of the 2190 RFU/min (IQR 1611–2660) measured in normal subjects. Similarly, the median phospholipid measured by XaCT was 438 ng/ml (IQR of 352–525) in MDS, compared to 885 ng/ml (IQR of 659–1035) in normal subjects.

3.3. MV characterisation - nanotracking analysis

In an effort to explain the lower ETP and phospholipid content of the MDS plasma, NTA was used to quantify the absolute number and size distribution of the MV it contained. Surprisingly, the distribution of MV in the size ranges 0–200 nm and 200–400 was similar between MDS and normal subjects as depicted in Fig. 2 (ANOVA one way for repeated measures, p > 0.05 for all comparisons).

In MDS, the median MV levels were noted to be 8.4×10^9/μl (IQR 6–19) in scatter and 4.4×10^9/μl (IQR 3.15–9.4) in fluorescent modes. The scatter mode was undertaken to evaluate all small particles (membrane bound and other particles) whilst the fluorescent mode (using Qdot 625) is specific for biological vesicles.[16]The median measurements for MV in normal subjects was similar - 8.4×10^9/μl (IQR of 1.8–18) in scatter and 7.3×10^9/μl (IQR of 2.6–10) in fluorescent mode. Nanotracking measurements were not significantly different between normal and MDS subjects for both scatter and fluorescent modes (see Fig. 2). There was no significant correlation observed between the MV levels (scatter or fluorescent) by nanotracking analysis and results obtained from functional assays (ETP and XaCT).

3.4. MV subsets in MDS - flow cytometry

Amongst the MV subtype levels tested, CD41, CD105, CD235, TF, annexin V and CD14 expressing MV were significantly lower in MDS compared to normal subjects (see Fig. 3 and Supplementary Table 2). The most common MV subtype in MDS was CD235 red cell MV with a median MV level of 22/μl (IQR of 13–45). On the other hand, CD41 expressing platelet MV were the most abundant MV in normal subjects and showed a median of 255/μl (interquartile range or IQR of 123–834/μl).

3.5. Small RNA cargo in MV from MDS

The quantities of small RNA and miRNA in MV from MDS patients were approximately twice that of the control plasma MV. In MDS, small RNA content was estimated to be 1037 pg/μl (IQR 6161–1921) and miRNA 297 pg/μl (123–581) respectively, which was significantly higher compared to small RNA 458 pg/μl (IQR 175–658) and miRNA content of 129 pg/μl (IQR 23–278) in normal subjects (p = 0.0005 and p = 0.02 respectively; as shown in Fig. 4). The miRNA normalised to mean MV was also compared and the miRNA content continued to be significantly higher in the MDS cohort (p = 0.014).

3.6. MV and correlation with blood counts clinical phenotype, bleeding and transfusion requirements in MDS

Correlation between MV subtypes/functional and blood counts showed that platelet counts correlated positively with platelet MV and inversely with WHO grade 3 or 4 bleeding scores respectively (Supplementary Table 3B). None of the other MV parameters correlated

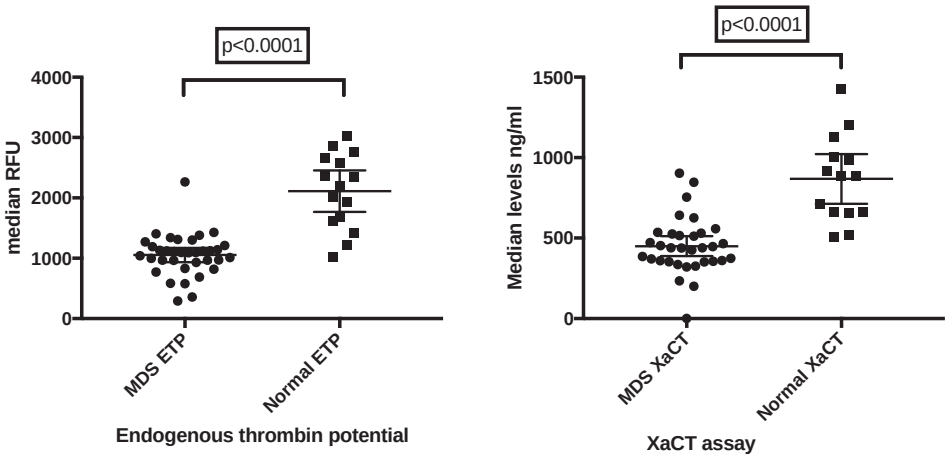

Fig. 1. Comparison of MV parameters by functional evaluation by ETP and XaCT assays between MDS (n = 35) and normal subjects (n = 15). The median ranks and 95% CI are depicted.

with blood counts or WHO grade 3 or 4 bleeding. A similar analysis was also performed in the normal subjects which did not show any significant correlations (Supplementary Table 3A).

The IPSS (international prognostic scoring system) and IPSS-R (in international prognostic scoring system- revised) risk scores were correlated with MV quantitation by flow cytometry, functional, NTA or miRNA analysis in MDS (see Supplementary Table 4). The IPSS-R score negatively correlated with CD235 red cell MV. It was also observed that PEAK thrombin, a functional MV parameter, correlated with IPSS-R and IPSS ($p = 0.048$ and $p = 0.039$ respectively). The XaCT test, which measures phospholipid MV contribution to coagulation, inversely correlated with IPPS score. The prognostic scores, the cytogenetic risk and severity of the WHO bleeding scores did not correlate with any other MV subsets, small RNA levels or MV levels measured by NTA analysis.

A subgroup analysis comparing MDS patients requiring red cell transfusions every 4 weeks or less (n = 5) compared with those requiring a red cell transfusion beyond 4 weeks (n = 4) was undertaken. These patients were selected as they all had platelets > 50 × 10^9/l and were not platelet transfusion dependent. No significant differences between the MV levels in these two groups of MDS by flow cytometry, functional, NTA analysis or small RNA cargo were observed.

3.7. miRNA expression analysis

The miRNA analysis showed a total of two mirs were under expressed whilst two others were over expressed. The differential expression specifically showed that mir-28 and mir-LET7D were under expressed whilst the mir-548J and mir-4485 were overexpressed in the MDS cohort compared to the normal cohort. The relation of these mirs to MDS and platelet activity was not known at the time of the analysis.

4. Discussion

MDS is a clonal bone marrow disorder associated with abnormal blood counts, transfusion dependence and risk of progression to leukemia. It is characterised by morphologically abnormal precursors in the bone marrow and increased apoptosis due to ineffective haemopoiesis. The evaluation of circulating MV in MDS has previously not been undertaken.

4.1. MV in MDS by functional coagulation, flow cytometry and NTA analysis

The bleeding diathesis of MDS is not completely explained by thrombocytopaenia. Our results show significantly lower procoagulant MV function by both ETP and XaCT tests, and lower CD41 MV (platelet derived) by flow cytometry compared to normal even when adjusted for platelet count. This is consistent with both a reduced production of large MV and reduced MV function. This explains a further part of the bleeding in MDS and presumably reflects the abnormal megakaryopoiesis that is often observed in MDS. This deficiency in 'procoagulant MV' in MDS is likely to be contributing to higher bleeding risk apart from the thrombocytopaenia alone.

However, in our small series, the variable nature of bleeding in our patients with MDS is not explained by the variation in CD41 MV as shown by the similarity of results in the 2 groups, one with increased blood transfusion requirements, and one without. As anticipated, platelet counts correlated with platelet MV and inversely with WHO grade

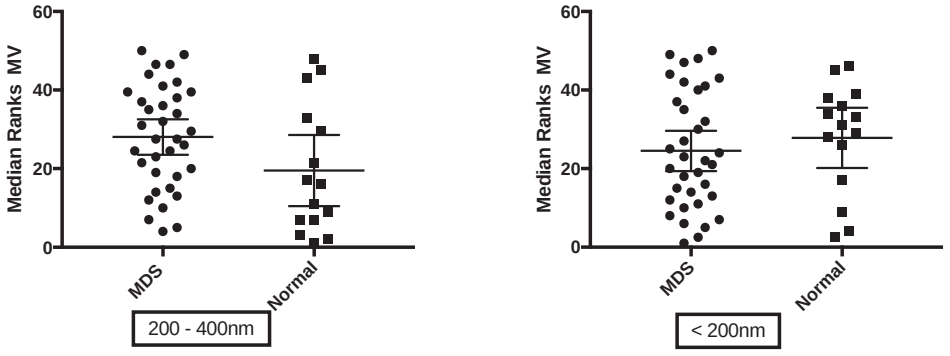

Fig. 2. Comparison of MDS (n = 35) and normal MV (n = 15) levels for 200–400 nm and < 200 nm using NTA in fluorescent mode. The median ranks and 95% CI are depicted.

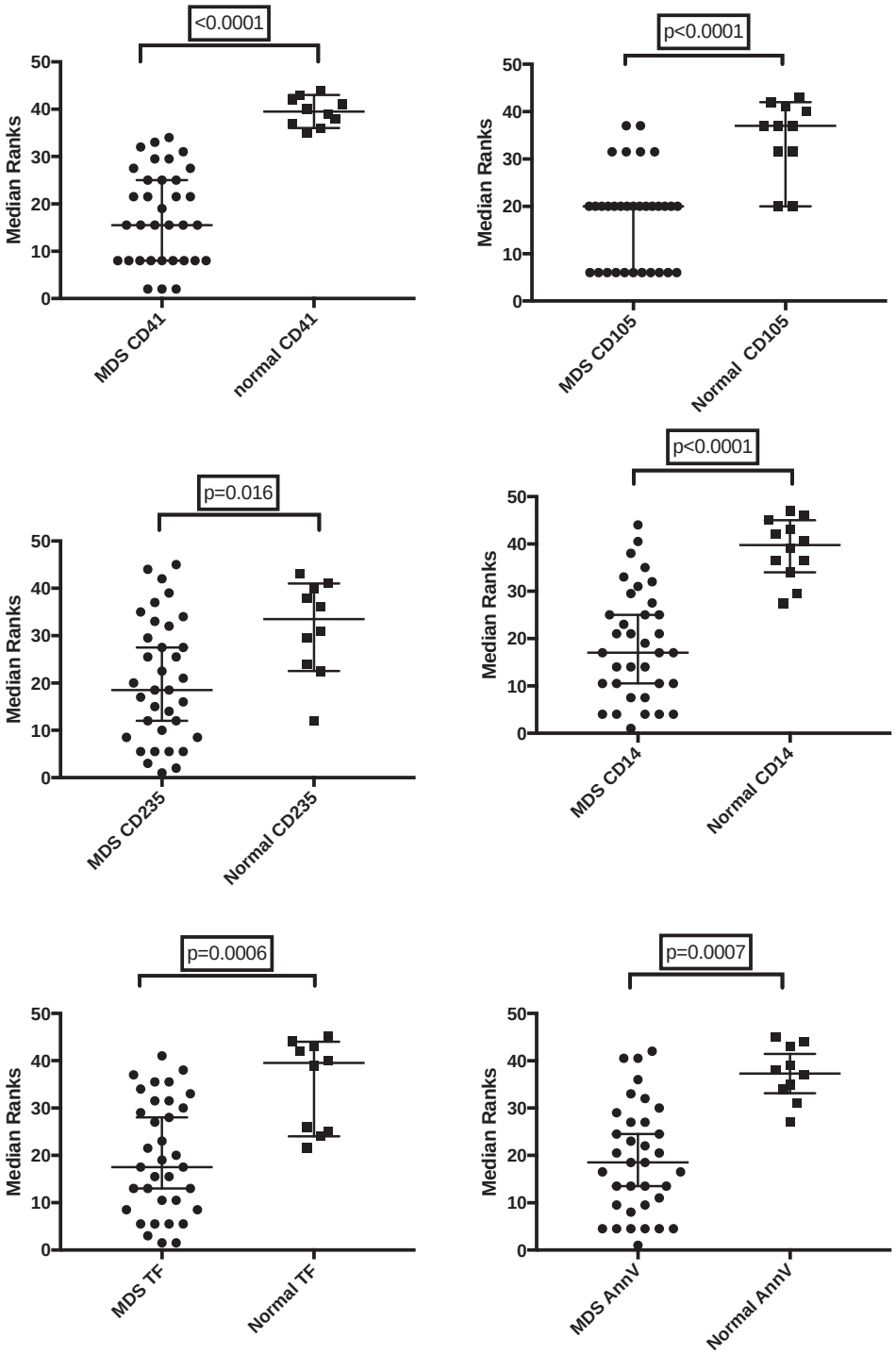

Fig. 3. Comparison of MV parameters by flow cytometry between MDS (n = 35) and normal subjects (n = 11). The median ranks and 95% CI are depicted.

3 or 4 bleeding scores respectively indicating that low platelet counts were associated with more clinically significant bleeding. In other studies, in non-MDS patients, the procoagulant function and flow cytometry results have been shown to correlate [17,18]. It is likely that the MV detected by flow is only the larger 'procoagulant functional' MV.

In MDS where there is significant apoptosis and abnormal megakaryocytes, it might have been expected that MV would be normal to increased. NTA measuring small MV shows normal numbers as predicted, but flow shows a marked reduction in large MV particularly platelet derived. This aspect will need further investigation with an increase in subject numbers to confirm these results and determine the origins of the smaller MV.

It could also be postulated that the MV levels/function in circulation do not completely reflect the MV generation in the bone marrow (BM). This study did not examine any BM samples. This may be technically difficult given the way BM is collected.

4.2. MV and correlation with MDS risk scores and frequency of red cell transfusions

The clinical risk scores (IPSS and IPSS-R) were correlated with the

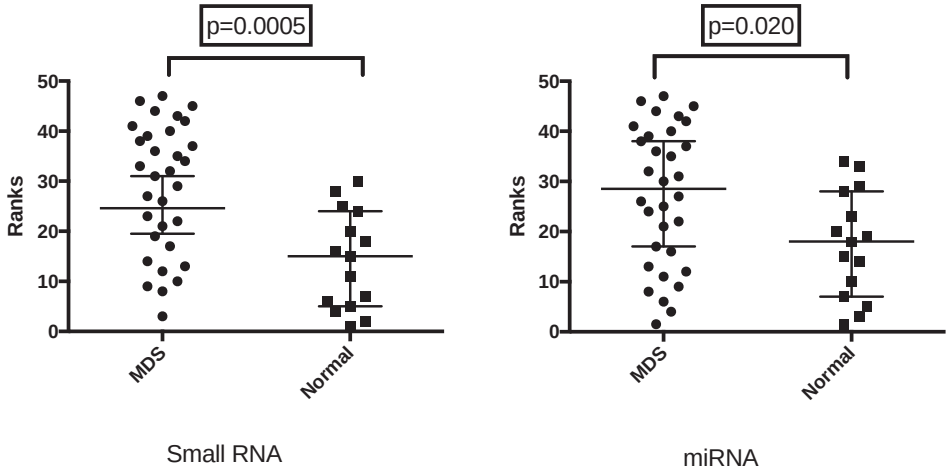

Fig. 4. Comparison of total small and miRNA isolated from MV between MDS (n = 35) and normal subjects (n = 15). The median ranks and 95% CI are depicted, normalised to small and miRNA obtained per 1 μl of MV pellet from 1.2 ml of plasma.

MV numbers and function. These clinical risk scores - IPSS and IPSS-R, are calculated on a combination of cytopaenias, bone marrow blast percentage and cytogenetic abnormalities. The IPSS-R, a more recent prognostic score, has cytopaenias defined in greater detail than IPSS [19]. The IPSS-R correlated negatively with CD235 MV as detected by flow cytometry indicating higher risk score was associated with lower red cell MV (Supplementary Table 4). In this study, there was a correlation between IPSS-R/IPSS and functional assessment of MV (PEAK thrombin and XaCT tests). However, the clinical significance of these observations is uncertain. A small subgroup analysis did not show any obvious differences in those who had a higher frequency of red cell transfusions. This finding needs to be evaluated in a larger cohort.

4.3. miRNA in MV

The total small RNA and miRNA content in MV was significantly higher in MDS patients compared to normal. In fact, the small RNA and miRNA contained were approximately twice that of the control plasma MV. These continued to be significantly higher in MDS even after normalisation to MV by NTA.

This study subsequently focussed on analysis of miRNA within MV as they have been shown to have an important role in transcription regulation and potentially transferrable between cells of the endovascular system even at distant sites [11,20]. We found that differential expression of several species of miRNA was different between the normal and MDS cohorts.

Specifically, mir-28 and mir-LET7D were under expressed. The mir-28 controls cell proliferation and post transcriptional gene silencing. It is reported to target the regulation of the thrombopoietin receptor, being overexpressed in patients with high platelets (in a subset of myeloproliferative disorders) [21]. Elevated mir-28 has also been reported to be been significantly associated with thrombosis/pulmonary embolism [22]. Under expression of mir-28 could therefore contribute to the thrombocytopaenia and/or bleeding by influencing platelet production/function and endothelial cell proliferation.

Interestingly mir-LET7D has been found in isolates of exosomal RNA in certain tissues and secretions (saliva and breast milk) though its functional annotation is not clear [23]. The detection of this mir-LET7D provides surrogate evidence that the miRNA isolated from MV in this study are indeed the species specific to MV and/or exosomes.

It was also observed that mir-548J and mir-4485 were over-expressed. Interestingly, mir-548J is associated with breast cancer cell invasion and metastasis [24]. Its role in myelodysplasia is not clear and needs evaluation in a larger cohort to study association with disease risk, progression and clinical behaviour. The role of mir-4485 has been reported to be under expressed in patients with chronic hepatitis and is reported as a marker for progression of liver disease; its role in MDS is not understood [25].

The small sample size and lack of a validation cohort for the RNA analysis are the main limitations of this study. In addition, the age of the normal cohort was lower than the MDS cohort and this was unavoidable given the few 'normal healthy' subjects above the age of 65 years who could be recruited into the study. This could have influenced MV results, however, previous studies have shown that older age cohorts (> 60 years) have a relatively uniform range of MV [26]. This study did not include any marrow samples for MV measurement to evaluate the differences between plasma and bone marrow MV but this would be a technically challenging exercise to undertake.

5. Conclusions

This analysis of MV in MDS shows significant differences in levels, function and small RNA content as compared to healthy normal controls. The lower levels of MV subtypes by flow cytometry and lower procoagulant function of MV in MDS appear to be a hallmark of this disease process. The clinical IPSS-R risk score correlated with the functional ETP parameter, however, quantitative or functional differences in MV did not correlate with level of transfusion dependence. The under and over expression of expression of miRNA in circulating MV in MDS is hypothesis generating and should be further explored.

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AKE, LL, MS and GI conceived the project; GI provided access to samples and in interpretation of data; MS provided access to coagulation testing and flow cytometry; AKE carried out the project and data analysis; AKE, LL, MS and GI were responsible for data interpretation and manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcmd.2018.11.001.

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5.5.5 Preliminary analysis of differential expression of small RNA and mitochondrial RNA

The total small RNA and miRNA content in MV was significantly higher in MDS patients compared to normal. A preliminary small RNA profile of RNA isolated from the MV was undertaken as part of this study. Differential expression showed a range of small RNA which was different between the normal and MDS cohorts. Two MDS samples had some similarities to the normal cohort – one of them had a low IPSS score of 0.5 and in the other a risk score could not be obtained due to a failed cytogenetic result. One striking feature was the under expression of certain mitochondrial RNA species in the MDS patients. A small number of miRNA were differentially expressed and this is explored further in the manuscript. The role of other small RNA which are also differentially expressed [see figure 5(d)] is unclear, as many of these small RNA species are not well annotated.

The intercellular transfer of mitochondrial components, including DNA or RNA, influences stem cell differentiation, cellular reprogramming and activation of inflammatory signalling. There is now experimental evidence that mitochondria transfer via a variety of mechanisms such as tunneling nanotubes, microvesicles, mitochondrial ejection and/or cytoplasmic fusion without recognising cellular boundaries.[123] This creates a previously unexplored pathway for cell to cell interaction and messaging.

More than 250 mitochondrial mutations have been described to be associated with disease.[123] Specifically, mtDNA (mitochondrial DNA) mutations in cancer appears to influence the bioenergetic and metabolic state of the cell – such acquired mitochondrial mutations have also been described in MDS.[124] The expression of mtRNA is also now recognised as important in both physiological and pathophysiological pathways.[125] Targeted mitochondrial instability in mice produced MDS like haematologic abnormalities suggesting a potential role for mitochondria in MDS pathophysiology.[126] There are also several other lines of evidence supporting this hypothesis including one study demonstrating decreased

mitochondrial gene expression across MDS phenotypes.[127] Treatment with drugs impairing mitochondrial protein synthesis (Chloramphenicol and Lenolizid) result in dyserythropoiesis.[121] In MDS with ring sideroblasts iron specifically accumulates in the mitochondria and there is widespread dyserythropoiesis in the marrow.[121,126] The under expression of mtRNA isolated from circulating MV is likely a reflection of the global changes that have occurred in the bone marrow environment. The exact role that this differential expression of mtRNA may play in the pathogenesis of MDS or its clinical phenotype is unclear at this stage. This observation is hypothesis generating. A KEGG (Kyoto encyclopaedia of genes and genomes) pathway analysis of the mtRNA and other differentially expressed small RNA is being planned. The differential expression results for mtRNA/ small RNA requires validation in a separate cohort before definitive conclusions can be made.

Horizontal transfer of material via EVs is an increasingly recognized phenomenon with important implications for tumorogenesis. In a model of colon cancer, EVs were able to induce a malignant phenotype in a non-malignant cell via such horizontal transfer. [128] Conversely, in another study blood platelets showed evidence for tumor derived RNA biomarkers. [129] Thus, studying miRNA content of MV/EV and how this interacts with neighbouring or distant tissue is very relevant to cancer biology.

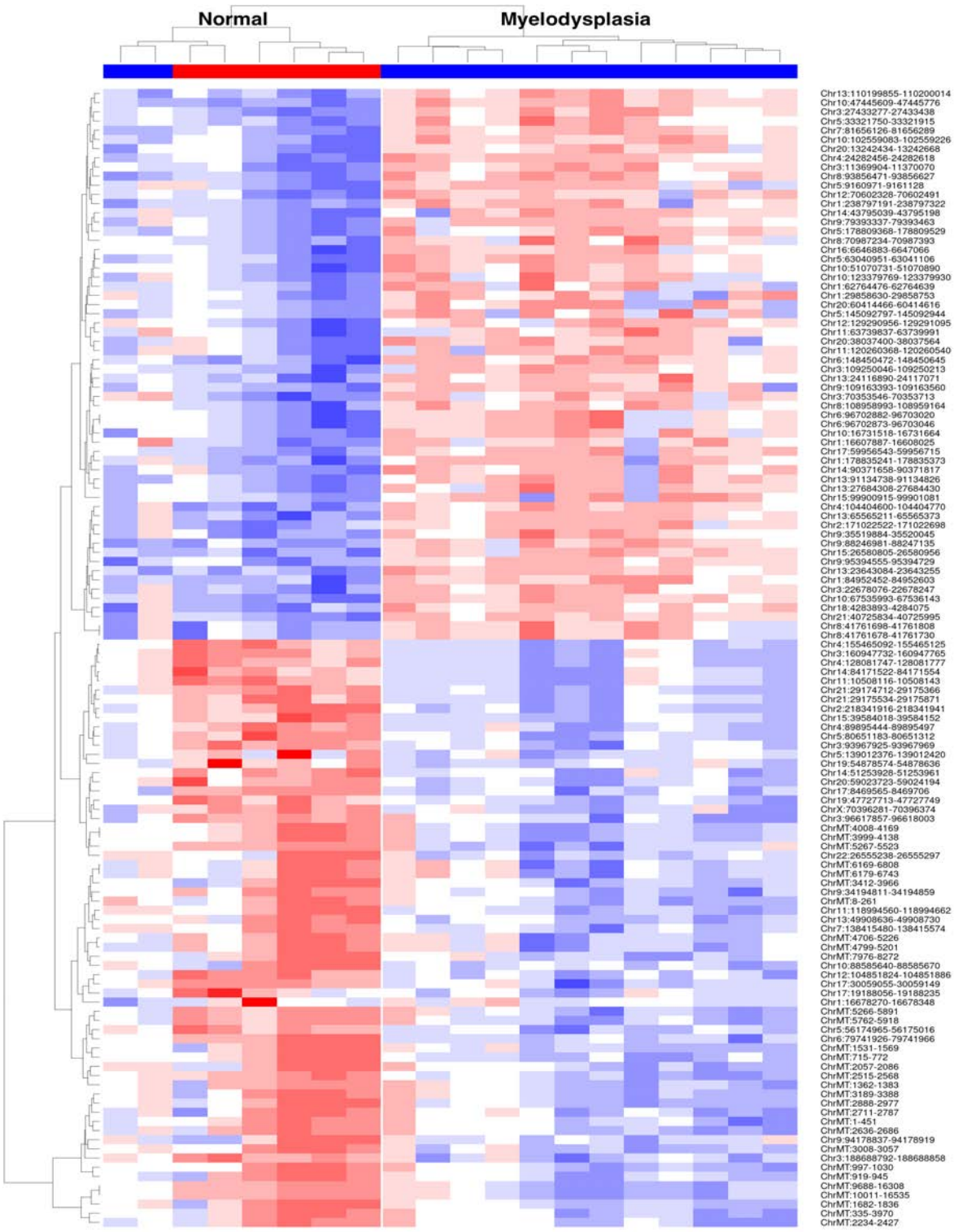

Figure 5(d). Differential expression of small RNA in Normal (left) and Myelodysplasia (right). Red bars indicate overexpression and blue bars indicate underexpression. White bars are areas where no difference in expression was detectable. Each row is a gene and each coloumn is a sample. The far right coloum represents the mapping to the human genome.

CHAPTER 6

CONCLUSIONS

"It has long been an axiom of mine that the little things are infinitely the most important."

Arthur Conan Doyle, **The Memoirs of Sherlock Holmes**

This thesis, its chapters and included publications, investigates,evaluates and discusses the following:

1) various approaches to investigate MV in human samples

2) novel optical and non-optical methods for analysing MV

3) evaluation of MV in normal healthy subjects

4) the role of MV in certain specific disease groups.

6.1 Outcomes and Significance

This research project aimed to look at several aspects of the role of MV all of which have been presented in previous chapters. The significance of these findings is summarized:

1. We analysed MV levels and associated biological variables in a cohort of normal healthy blood donors. This work provided baseline information on healthy subjects as well as interpretation of MV levels in diseased subjects. This work would help researchers ensure biological variables are considered when choosing controls for their MV experiments. It also reinforces that preanalytical variables need to be similar across controls and patient samples and a range of techniques should be used to evaluate MV.

2. We investigated two new technologies for MV assessment - TRPS and NTA. These techniques are sensitive even in the range of 100nm or below. However, they evaluate slightly different size ranges and understanding the advantages as well disadvantages of each approach helps to choose the best technique for the experimental question. Correlation of novel techniques with existing methods such as flow cytometry and functional clot based studies provided information on what techniques could be best employed to detect MV in a given clinical study. Understanding the pros and cons of every technique for studying MV enables the choice of the most apt method to analyse the feature of MV we are most interested in.

3. The role of MV in haemostasis and thrombosis was investigated in factor V Leiden subjects as well as in snake bite envenomation. These clinical cohorts provided the unique opportunity to evaluate the role of MV in the pathogenesis of thrombosis (Factor V Leiden subjects) and bleeding (snake bite patients).

4. The factor V Leiden cohort showed increased MV levels in both homozygotes and heterozygotes that did not correlate with occurrence of thrombosis. This work was followed up by Dr Lisa Lincz (research supervisor) which led to a subsequent publication looking at variability in plasma factor V levels and this seemed to correlate with platelet MV.[130]

5. Plasma MV from snake bite patients were evaluated for correlation with and as a potential marker for cell specific toxin mediated endothelial cell damage. Further consideration is being given by the research team on how to develop ex vivo models to confirm this initial finding.

6. The final chapter is the first report of circulating levels and function of MV in Myelodysplasia in published literature. The levels/function for circulating MV were

markedly reduced but the total small RNA content was raised amongst the MDS cohort compared to controls. The miRNA isolated from circulating MV in MDS differed from normal controls – this is likely to be a true biological finding but technical challenging experiment that will need confirmation. Further evaluation of the role other noncoding RNA, such as mitochondrial miRNA in MDS is also warranted as these were significantly under expressed in the myelodysplasia cohort.

6.2 Pitfalls and barriers

There were several barriers to undertaking the project and these were faced at different time points. These are described in brief below both as a reflective process and as a resource for any future work undertaken.

1. Experimental techniques: The measurement of MV function, numbers and miRNA content is a technically challenging area with a number of developments in the area of nanoscale detection which are now occurring. Both the PhD student and one research supervisor (Dr. Lisa Lincz) have been regular participants in workshops and meetings for standardization of measurement of MV conducted by the ISTH. This enabled the understanding of evolving technologies and how to apply some of these techniques to the evaluation of MV.

2. Several research plans had to be altered or techniques changed when the results were not those expected. For example, when the low quantities of miRNA were obtained from MV it was decided to undertake the miRNA sequencing external to our laboratory as the technology to undertake the sequencing locally at such low quantities of input material was not available. Although, there was an initial plan to study the effect of MV from normal or diseased subjects on endothelial cell growth in vitro, it was decided not to

pursue this line of activity given the wide variation in the function as well numbers of MV from patient samples.

3. Funding and research time challenges: The experimental work of the candidate was in general well-funded. All equipment and reagents required were funded by either local research grants and/or private practise trust funds support. However, dedicated time for research was a major constraint as this was undertaken part-time in addition to clinical duties. The Hunter Cancer research Alliance (HCRA) fellowship as well as clinical research fellowship (Hunter New England Health/Pathology North- Hunter/HCRA and Calvary Mater joint funding) enabled protected time for research and led to completion of the projects planned.

4. Limitations of the work: The work focussed on technical aspects of MV isolation and characterization in the first half of the study. This technical focus meant that majority of the initial publications were methodology based (Chapters 2 and 3). The second part of the project involved cohort studies (Chapters 4 and 5). Key findings in the MDS cohort would benefit from further experiments to validate these findings in a larger cohort of patients.

6.3 Conclusions and future directions

As the role of MV in biological and pathophysiological conditions emerge, a role for measuring the MV may assume a diagnostic or prognostic significance. At this point time, the study of MV provides another path into understanding the vascular biology and interaction of various blood cells. The exploration of new ideas is a journey and not a destination. An investigatory journey as in this thesis unfolds some of these truths, exposes challenges in interpreting these truths and brings up many more questions during the sojourn. A thorough knowledge of the tools measuring MV and then its application in the

study of MV in a range of disorders provides this first step. Future steps include the exploration of the effect of MV on angiogenesis using culture models and the effect of drugs on endothelial damage where MV may play a significant role. The dream of every researcher is translation of their research into clinical practise and eventual patient benefit. The investigation of the role of miRNA in MV using cell culture models has the potential to shed further light on their role in regulating cellular function (haemopoiesis and angiogenesis) in Myelodysplasia. Small molecular silencers of miRNA could then be employed to modulate their function and bring about the desired effect to improve outcomes in such diseases. As I yearn for awe, wonder and learning that awaits me, I start again at the end of the beginning…….

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APPENDIX

Differential expression analysis algorithm
Ethics approvals
Informed consent
Statements of contributions from co-authors and endorsement by Faculty Assistant Dean (Research and Training)

microRNA in vesicle, AML

Project:	NGS Analysis of extracellular vesicle microRNA in AML
ProjectID:	B18007
Type:	Data analysis project
Date:	22-May-2018
CI(s):	Dr. Anoop Enjeti

Data

Data consists of single end 76bp long reads, on 30 samples (10 normal / controls and 20 MDS) for smallRNA in extracellular vesicle in blood plasma.

Analysis methodology

Preprocessing

Pre-processing consits of adapter identification and removal, plus quality removal. Reads shorter than 18bp after trimming are removed. General statistics about preprocessing and QC in Table 3.

Analysis

1. **Method 1:** Consisted of:

a. aligning to reference genome GRCh38. Bowtie2 with parameters for small RNA alignment.
b. feature counts based on miRBase microRNA annotation v.22 (20180305)
c. differential expression analysis with DESeq2

2. **Method 2:** Consisted of:

a. removal of low complexity reads based on DUST algorithm
b. mapping reads to small non-coding RNA databases via BLAST
c. counting reads per feature
d. differential expression analysis on feature counts

None of the analysis above yield any significant results when applied to the full set of samples.

Results method 1

General alignment statistics of method 1 are given in Table 4, while the statistics on the mapping to the (human) micro RNA database in Table 5. Reads with multiple mappings to genome ("ambiguous reads") have been counted only towards the first mapping encountered, while reads mapping to multiple features that overlap count once for each overlapping feature (column r.ambig2 in Table 5).

Results method 2

The second method uses a wider set of small RNA features and attempts to directly map as much as possible to RNA sequences of known features without relying on genomic mapping. It is therefore quite independent of the "traditional" reference genome alignment (but features are still consensus features from the databases).

Global statistics for this method are given in Table 6. The relative proportion of different small RNA species for each sample is depicted in Figure 1, while the absolute values are plotted on a common scale in Figure 2. There is a large variability among samples, prompting to investigate outliers more in detail.

```
## Using sample as id variables
```

```
## Using sample as id variables
```

Refinement

Based on the smallRNA species composition and quality control data from the samples, plus hierarchical clustering based on raw counts, a subset of samples are further selected for analysis.

Samples 4, 29 and 30 are excluded from the Control group due to the higher than 75% proportion of unknown / unmapped reads to identified features. Control sample 8 is also excluded due to the very high proportion of genic ribosomal RNA and high unknown proportion.

Samples 16, 20, 21 from the MDS group are excluded due to high unknown proportion, and samples 9, 10 and 15 due to combined ribosomal plus unknown proportion.

Data is quantized for differential expression defining as *contig probes*: any region longer than 20bp which contains at least 2 reads in the aggregated samples, with a maximum gap of 20bp. Differential expression is computed by two read count based methods. It is worth noticing these quantisation is not associated to known small RNA features, but will define a feature as long as there are reads mapping to that particular genomic region, strand specific. Annotation to known features is performed on the significant differential expression results, if any.

Chr	Start	End		Length	FDR	FDR.2	Feature	ID		Type	Orientation	Dist
1	16607887	16608025	-	139	0.016		NBPF1	ENSG00000219481	-	gene	overlapping	0
1	16678270	16678348	-	79	0.035							0
1	29858630	29858753	-	124	0.037	0.050						0
1	62764476	62764639	-	164	0.014	0.022						0
1	84952452	84952603	-	152		0.005	MCOLN2	ENSG00000153898	-	gene	overlapping	0
1	178835241	178835373	-	133	0.023	0.010	RALGPS2	ENSG00000116191	+	gene	overlapping	0
1	238797191	238797322	-	132		0.030						0
10	16731518	16731664	-	147	0.025		RSU1	ENSG00000148484	-	gene	overlapping	0
10	47445609	47445776	-	168	0.050	0.017						0
10	51070731	51070890	+	160	0.000	0.002	PRKG1	ENSG00000185532	+	gene	overlapping	0
10	67535993	67536143	-	151	0.000	0.000	CTNNA3	ENSG00000183230	-	gene	overlapping	0
10	88585640	88585670	-	31	0.001	0.000	Y_RNA	ENSG00000201548	-	gene	overlapping	0
10	102559083	102559226	-	144	0.012	0.023	SUFU	ENSG00000107882	+	gene	overlapping	0
10	123379769	123379930	-	162	0.036		AL160290.2	ENSG00000230131	+	gene	overlapping	0
11	10508116	10508143	-	28	0.034		MIR4485-201	ENST00000638094	-	miRNA	upstream	127
							MTRNR2L8	ENSG00000255823	-	gene	overlapping	0
11	63739837	63739991	+	155	0.019		RTN3	ENSG00000133318	+	gene	overlapping	0
11	118994560	118994662	+	103	0.011	0.033	Metazoa_SRP	ENSG00000280502	+	gene	overlapping	0
11	120260368	120260540	+	173	0.034		POU2F3	ENSG00000137709	+	gene	overlapping	0
12	70602328	70602491	-	164	0.014	0.023	PTPRB	ENSG00000127329	-	gene	overlapping	0
12	104851824	104851886	+	63	0.000	0.000	SLC41A2	ENSG00000136052	-	gene	overlapping	0
12	129290956	129291095	+	140	0.007		TMEM132D	ENSG00000151952	-	gene	overlapping	0
13	23643084	23643255	-	172	0.018	0.001	TNFRSF19	ENSG00000127863	+	gene	overlapping	0
13	24116890	24117071	+	182	0.036		AL359736.1	ENSG00000273167	+	gene	overlapping	0
13	27684308	27684430	-	123		0.026	POLR1D	ENSG00000186184	+	gene	overlapping	0
13	49908636	49908730	-	95	0.001	0.001	RNY4P9	ENSG00000200064	-	gene	overlapping	0
13	65565211	65565373	-	163	0.001	0.000						0
13	91134738	91134826	-	89		0.019						0
13	110199855	110200014	-	160		0.047	COL4A1	ENSG00000187498	-	gene	overlapping	0
14	43795039	43795198	+	160	0.005	0.032						0
14	51253928	51253961	-	34	0.012		TMX1	ENSG00000139921	+	gene	overlapping	0
14	84171522	84171554	+	33	0.007							0
14	90371658	90371817	-	160		0.023						0
15	26580805	26580956	-	152		0.002	GABRB3	ENSG00000166206	-	gene	overlapping	0
15	39584018	39584152	+	135	0.022		THBS1	ENSG00000137801	+	gene	overlapping	0
15	99900915	99901081	-	167		0.021						0
16	6646883	6647066	+	184	0.005		RBFOX1	ENSG00000078328	+	gene	overlapping	0
17	8469565	8469706	-	142	0.000	0.000	NDEL1	ENSG00000166579	+	gene	overlapping	0
17	19188056	19188235	+	180	0.000	0.000	SNORD3A-201	ENST00000584923	+	snoRNA	overlapping	0
							SNORD3A	ENSG00000263934	+	gene	overlapping	0
17	30059055	30059149	-	95	0.000	0.007	EFCAB5	ENSG00000176927	+	gene	overlapping	0
17	59956543	59956715	-	173	0.006	0.001	AC005702.2	ENSG00000267318	+	gene	overlapping	0
18	4283893	4284075	-	183		0.042	DLGAP1	ENSG00000170579	-	gene	overlapping	0
19	47727713	47727749	-	37	0.004		AC010519.1	ENSG00000268746	+	gene	overlapping	0
19	54878574	54878636	-	63	0.021	0.000	FCAR	ENSG00000186431	+	gene	overlapping	0
2	171022522	171022698	-	177		0.007	TLK1	ENSG00000198586	-	gene	overlapping	0
2	218341916	218341941	+	26	0.026	0.047	MIR6810-201	ENST00000622701	+	miRNA	overlapping	0
							PNKD	ENSG00000127838	+	gene	overlapping	0
20	13242434	13242668	-	235	0.015	0.019	ISM1	ENSG00000101230	+	gene	overlapping	0
20	38037400	38037564	+	165	0.036		RPRD1B	ENSG00000101413	+	gene	overlapping	0
20	59023723	59024194	+	472	0.000	0.002	TUBB1	ENSG00000101162	+	gene	overlapping	0
20	60414466	60414616	-	151		0.030	MIR646HG	ENSG00000228340	+	gene	overlapping	0
21	29174712	29175366	+	655	0.011	0.025	MAP3K7CL	ENSG00000156265	+	gene	overlapping	0

21	29175534	29175871	+	338	0.034		MAP3K7CL	ENSG00000156265	+	gene	overlapping	0
21	40725834	40725995	-	162	0.000	0.001	DSCAM	ENSG00000171587	-	gene	overlapping	0
22	26555238	26555297	-	60		0.024	MIR548J-201	ENST00000408833	-	miRNA	overlapping	0
							TPST2	ENSG00000128294	-	gene	overlapping	0
3	11369904	11370070	+	167	0.005		ATG7	ENSG00000197548	+	gene	overlapping	0
3	22678076	22678247	-	172	0.015	0.000						0
3	27433277	27433438	-	162	0.016	0.004	SLC4A7	ENSG00000033867	-	gene	overlapping	0
3	70353546	70353713	+	168	0.035		SAMMSON	ENSG00000240405	+	gene	overlapping	0
3	93967925	93967969	+	45	0.022		PROS1	ENSG00000184500	-	gene	overlapping	0
3	96617857	96618003	-	147	0.007		MTRNR2L12	ENSG00000269028	-	gene	overlapping	0
3	109250046	109250213	-	168	0.038							0
3	160947732	160947765	+	34	0.003		PPM1L	ENSG00000163590	+	gene	overlapping	0
3	188688792	188688858	+	67	0.020	0.020	MIR28-201	ENST00000384918	+	miRNA	overlapping	0
							LPP	ENSG00000145012	+	gene	overlapping	0
4	24282456	24282618	+	163	0.012							0
4	89895444	89895497	+	54	0.001	0.020	MMRN1	ENSG00000138722	+	gene	overlapping	0
4	104404600	104404770	-	171	0.001	0.000	AC004052.1	ENSG00000251170	+	gene	overlapping	0
4	128081747	128081777	-	31	0.005		LARP1B	ENSG00000138709	+	gene	overlapping	0
4	155465092	155465125	-	34	0.009							0
5	9160971	9161128	-	158	0.009		SEMA5A	ENSG00000112902	-	gene	overlapping	0
5	33321750	33321915	-	166	0.035	0.004						0
5	56174965	56175016	-	52	0.001	0.001	ANKRD55	ENSG00000164512	-	gene	overlapping	0
5	63040951	63041106	+	156	0.003							0
5	80651183	80651312	-	130	0.003	0.047	DHFR	ENSG00000228716	-	gene	overlapping	0
5	139012376	139012420	+	45	0.010		SIL1	ENSG00000120725	-	gene	overlapping	0
5	145092797	145092944	-	148		0.050						0
5	178809368	178809529	-	162	0.039		AACSP1	ENSG00000250420	-	gene	overlapping	0
6	79741926	79741966	+	41	0.000	0.000						0
6	96702873	96703046	+	174	0.014							0
6	96702882	96703020	-	139	0.014							0
6	148450472	148450645	+	174	0.013		SASH1	ENSG00000111961	+	gene	overlapping	0
7	81656126	81656289	-	164	0.039	0.018	AC008163.1	ENSG00000233491	-	gene	overlapping	0
7	138415480	138415574	+	95	0.003	0.006						0
8	41761678	41761730	+	53		0.020	ANK1	ENSG00000029534	-	gene	overlapping	0
8	41761698	41761808	-	111		0.019	ANK1	ENSG00000029534	-	gene	overlapping	0
8	70987234	70987393	-	160		0.020						0
8	93856471	93856627	-	157		0.021						0
8	108958993	108959164	-	172	0.014		AC104248.1	ENSG00000253796	-	gene	overlapping	0
9	34194811	34194859	-	49	0.001	0.001	UBAP1	ENSG00000165006	+	gene	overlapping	0
9	35519884	35520045	-	162		0.032	RUSC2	ENSG00000198853	+	gene	overlapping	0
9	79393337	79393463	+	127	0.000	0.010						0
9	88246981	88247135	-	155		0.043						0
9	94178837	94178919	+	83	0.023	0.013	MIRLET7D-201	ENST00000362263	+	miRNA	overlapping	0
							AL158152.2	ENSG00000269946	+	gene	overlapping	0
9	95394555	95394729	-	175		0.031	FANCC	ENSG00000158169	-	gene	overlapping	0
9	109163393	109163560	+	168	0.031		FRRS1L	ENSG00000260230	-	gene	overlapping	0
MT	1	451	-	451	0.000	0.000	MT-TF	ENSG00000210049	+	gene	downstream	126
MT	8	261	+	254	0.020	0.021	MT-TF	ENSG00000210049	+	gene	downstream	316
MT	335	3970	+	3636	0.000	0.000	MT-TF	ENSG00000210049	+	gene	overlapping	0
MT	715	772	-	58	0.000	0.000	MT-RNR1	ENSG00000211459	+	gene	overlapping	0
MT	919	945	-	27	0.000	0.000	MT-RNR1	ENSG00000211459	+	gene	overlapping	0
MT	997	1030	-	34	0.000	0.000	MT-RNR1	ENSG00000211459	+	gene	overlapping	0
MT	1362	1383	-	22	0.034	0.021	MT-RNR1	ENSG00000211459	+	gene	overlapping	0
MT	1531	1569	-	39	0.000	0.000	MT-RNR1	ENSG00000211459	+	gene	overlapping	0
MT	1682	1836	-	155	0.000	0.000	MT-RNR2	ENSG00000210082	+	gene	overlapping	0
MT	2057	2086	-	30	0.014		MT-RNR2	ENSG00000210082	+	gene	overlapping	0
MT	2234	2427	-	194	0.002	0.001	MT-RNR2	ENSG00000210082	+	gene	overlapping	0
MT	2515	2568	-	54	0.000	0.000	MT-RNR2	ENSG00000210082	+	gene	overlapping	0
MT	2636	2686	-	51	0.001	0.000	MT-RNR2	ENSG00000210082	+	gene	overlapping	0
MT	2711	2787	-	77	0.003	0.001	MT-RNR2	ENSG00000210082	+	gene	overlapping	0
MT	2888	2977	-	90	0.001	0.000	MT-RNR2	ENSG00000210082	+	gene	overlapping	0
MT	3008	3057	-	50	0.001	0.000	MT-RNR2	ENSG00000210082	+	gene	overlapping	0
MT	3189	3388	-	200	0.007	0.007	MT-RNR2	ENSG00000210082	+	gene	overlapping	0
MT	3412	3966	-	555	0.000	0.000	MT-ND1	ENSG00000198888	+	gene	overlapping	0
MT	3999	4138	-	140	0.011	0.000	MT-ND1	ENSG00000198888	+	gene	overlapping	0
MT	4008	4169	+	162	0.013	0.001	MT-ND1	ENSG00000198888	+	gene	overlapping	0
MT	4706	5226	-	521	0.003	0.001	MT-ND2	ENSG00000198763	+	gene	overlapping	0
MT	4799	5201	+	403	0.002	0.001	MT-ND2	ENSG00000198763	+	gene	overlapping	0
MT	5266	5891	-	626	0.000	0.001	MT-ND2	ENSG00000198763	+	gene	overlapping	0
MT	5267	5523	+	257	0.013	0.021	MT-ND2	ENSG00000198763	+	gene	overlapping	0
MT	5762	5918	+	157	0.000	0.000	MT-TC	ENSG00000210140	-	gene	overlapping	0
MT	6169	6808	+	640	0.021	0.026	MT-CO1	ENSG00000198804	+	gene	overlapping	0
MT	6179	6743	-	565	0.020	0.024	MT-CO1	ENSG00000198804	+	gene	overlapping	0
MT	7976	8272	-	297	0.002	0.000	MT-CO2	ENSG00000198712	+	gene	overlapping	0
MT	9688	16308	+	6621	0.005	0.010	MT-CO3	ENSG00000198938	+	gene	overlapping	0
MT	10011	16535	-	6525	0.005	0.010	MT-TG	ENSG00000210164	+	gene	overlapping	0
X	70396281	70396374	+	94	0.034		KIF4A	ENSG00000090889	+	gene	overlapping	0

Table 2: Differential expression significant results. Data is sorted by genomic position. Table show consensus between two dXp methods, indicated by the FDR value in columns **FDR** (Method 1 – 'edgeR') and **FDR.2** (Method 2 – 'DESeq2'). FDRs denoted as '0.000' are one or two decimal places to the right, the minimum FDR values are: FDR: 8.44×10^{-6}, FDR.2: 1.14×10^{-6}. Quantitation is by contig features, annotations indicate miR, snoRNA, snRNA and genes up to 1kbp from either side of contig.

```
##
## Attaching package: 'gplots'

## The following object is masked from 'package:stats':
##
##     lowess
```

Annotated list of probes found significant in the refined analysis using algorithm 1 (DESeq2) is in **??**, and **??** gives the list found using algorithm 2 (edgeR). The correspondence is high, the overlap between methods is on the order of 70%.

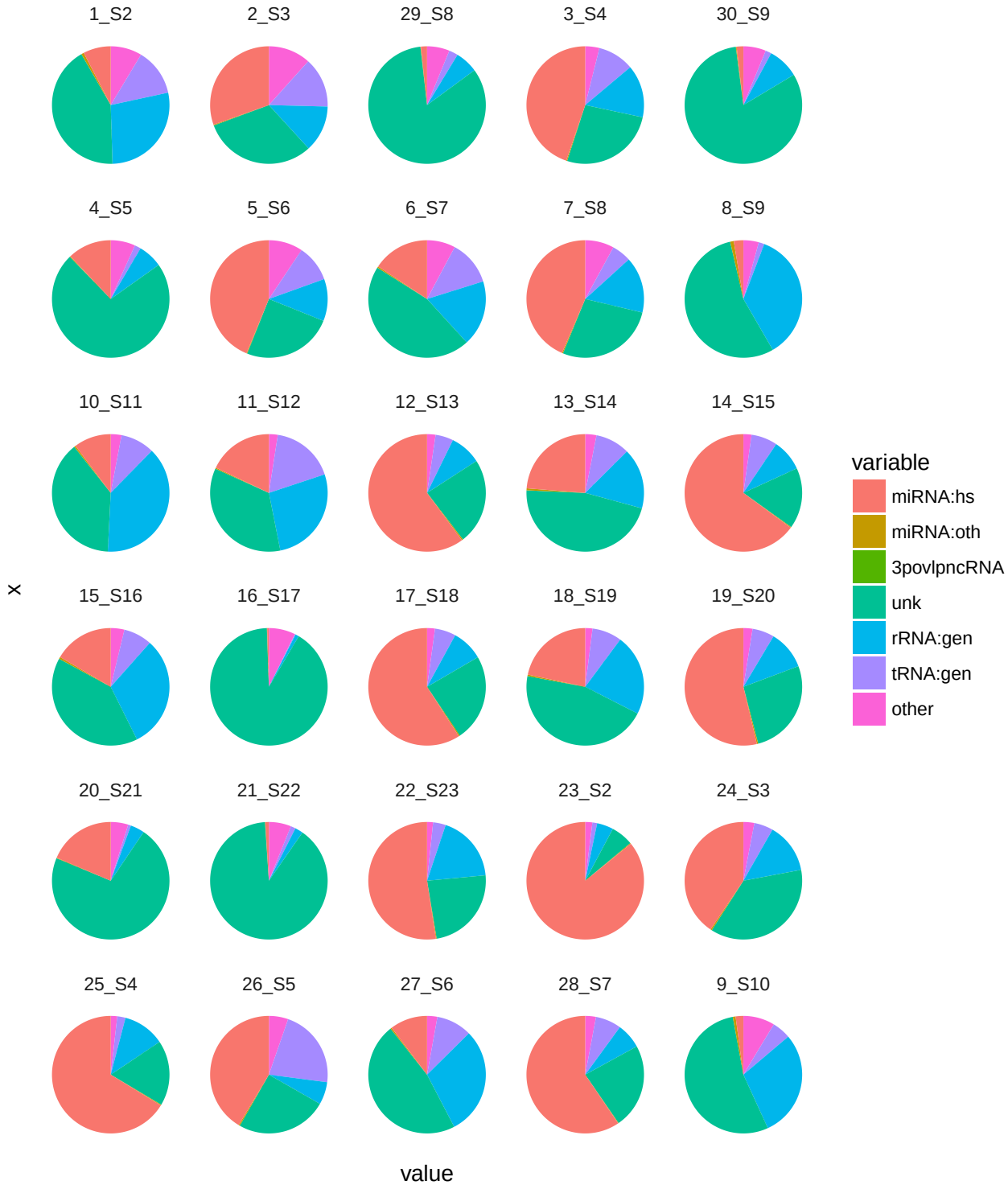

Figure 1: Proportion of different smallRNA species

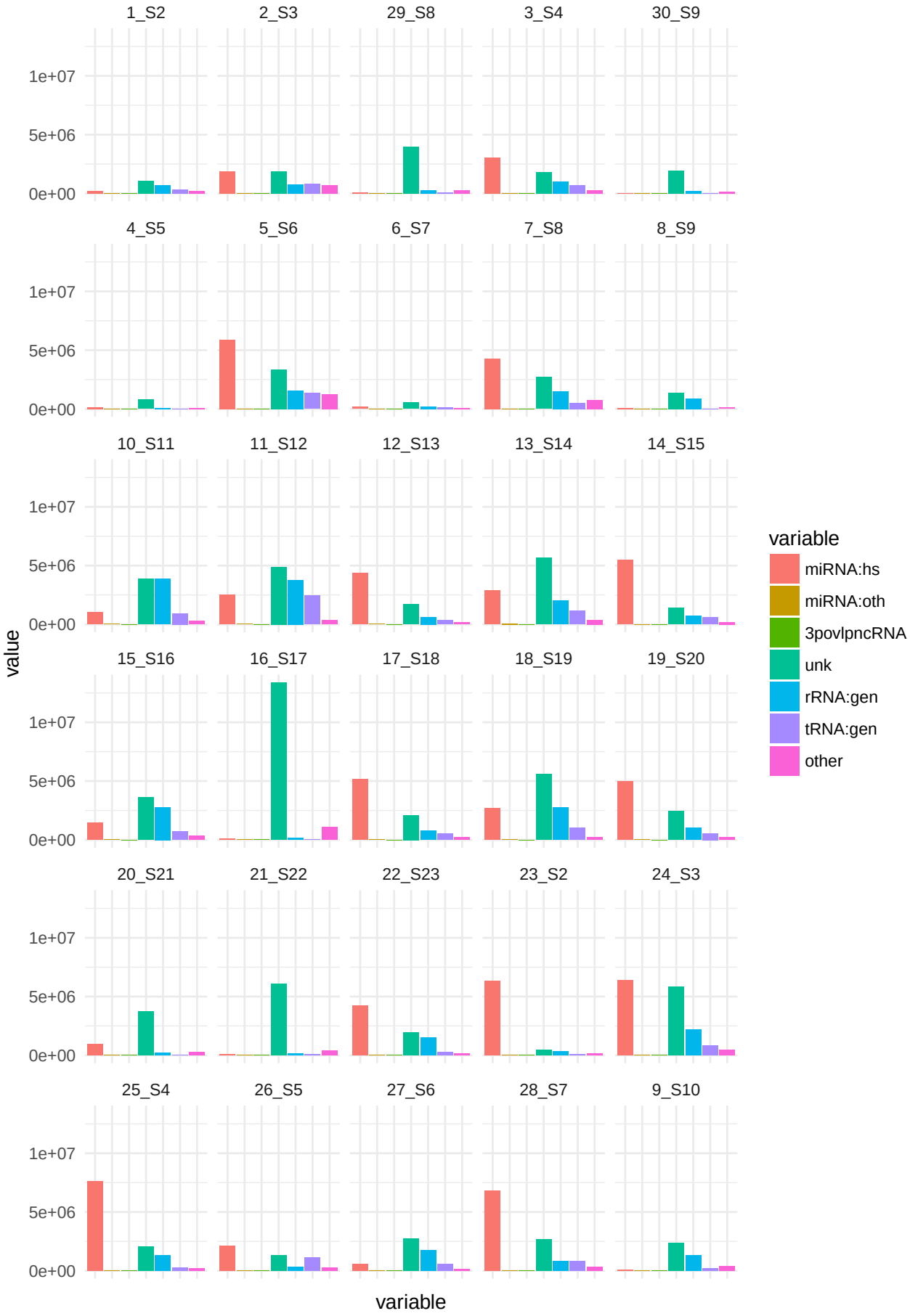

Figure 2: Number of reads for different species

5

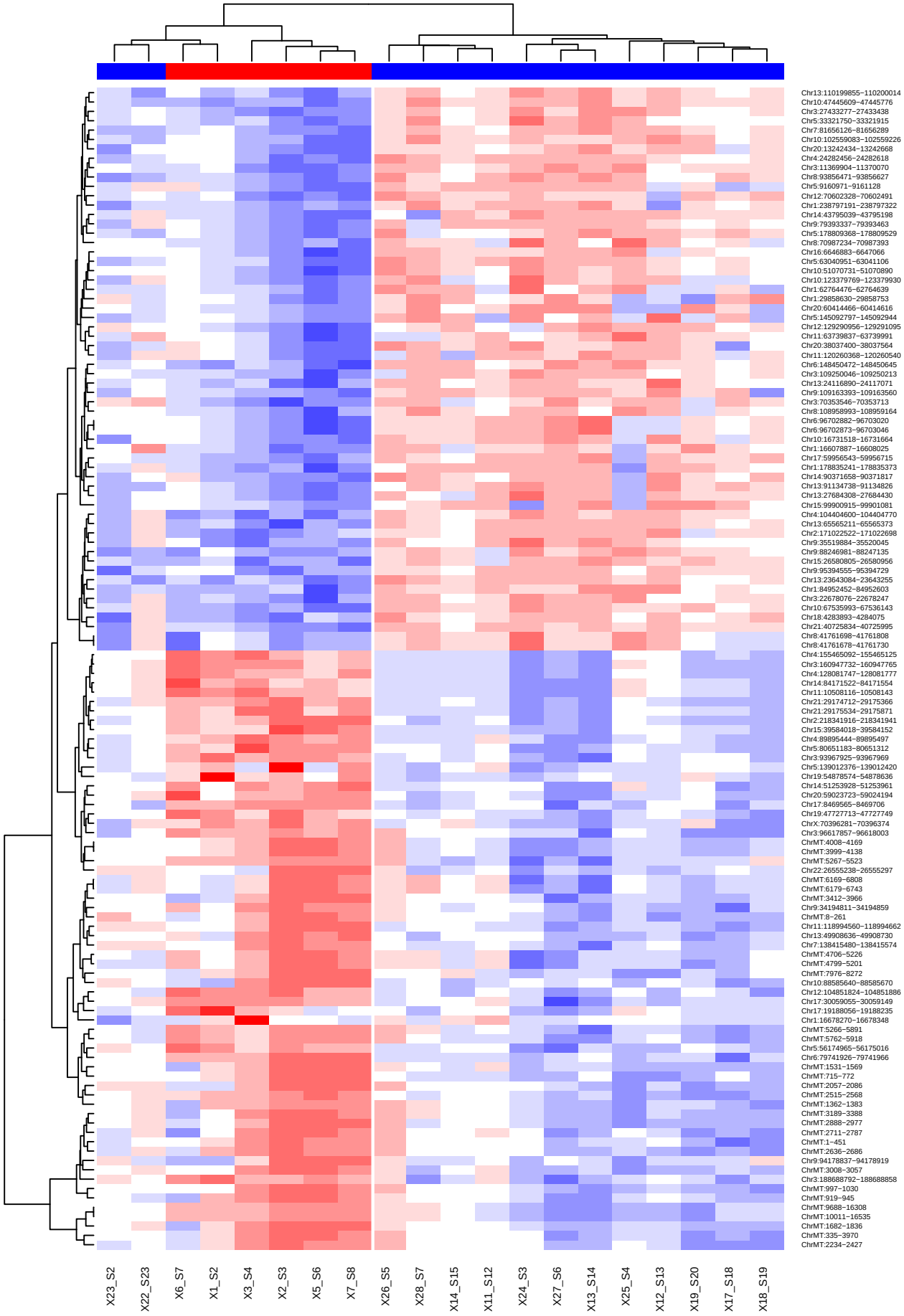

Figure 3: Heatmap of small RNA expression values.

Subsequent analysis

The following would be worth attempting to improve DE results

- Investigate source of non-human small RNA in the high proportion samples.
- More lenient mapping not discarding multiple best alignments, and differential expression on those.
- One more sample should be removed as outlier, based on clustering of samples.

Based on DE results, investigate what the un-annotated features are: sequence correspondence to other miRNA families or other small RNA molecules.

Appendix

% latex table generated in R 3.4.3 by xtable 1.8-2 package % Tue May 22 13:06:35 2018

sample	case	r.total	r.adapt	r.pass	r.short	r.final	pc.adapt	pc.pass	pc.short
87119-Sample_1_S2_R1	N	8339803	7888581	8339803	1975820	6363983	94.6	100.0	23.7
87119-Sample_2_S3_R1	N	14011806	13366862	14011806	430784	13581022	95.4	100.0	3.1
87119-Sample_29_S8_R1	N	21676901	20096448	21676901	6897286	14779615	92.7	100.0	31.8
87119-Sample_3_S4_R1	N	13218701	12458876	13218701	1201124	12017577	94.3	100.0	9.1
87119-Sample_30_S9_R1	N	13259829	12577804	13259829	7229260	6030569	94.9	100.0	54.5
87119-Sample_4_S5_R1	N	5432792	5158669	5432792	2213732	3219060	95.0	100.0	40.7
87119-Sample_5_S6_R1	N	24274906	23076369	24274906	538464	23736442	95.1	100.0	2.2
87119-Sample_6_S7_R1	N	5900943	5700528	5900943	1996743	3904200	96.6	100.0	33.8
87119-Sample_7_S8_R1	N	25465636	24035655	25465636	1382038	24083598	94.4	100.0	5.4
87119-Sample_8_S9_R1	N	17998137	17473477	17998137	4298490	13699647	97.1	100.0	23.9
87119-Sample_10_S11_R1	Y	17216487	14871694	17216487	1746318	15470169	86.4	100.0	10.1
87119-Sample_11_S12_R1	Y	19620729	17048438	19620729	1069413	18551316	86.9	100.0	5.5
87119-Sample_12_S13_R1	Y	15921186	14589601	15921186	904117	15017069	91.6	100.0	5.7
87119-Sample_13_S14_R1	Y	22321194	20044472	22321194	2228369	20092825	89.8	100.0	10.0
87119-Sample_14_S15_R1	Y	16707528	16017823	16707528	1076746	15630782	95.9	100.0	6.4
87119-Sample_15_S16_R1	Y	15393390	13493110	15393390	902515	14490875	87.7	100.0	5.9
87119-Sample_16_S17_R1	Y	42455189	38863514	42455189	21128085	21327104	91.5	100.0	49.8
87119-Sample_17_S18_R1	Y	17581212	16498260	17581212	1066368	16514844	93.8	100.0	6.1
87119-Sample_18_S19_R1	Y	16491868	13605037	16491868	875577	15616291	82.5	100.0	5.3
87119-Sample_19_S20_R1	Y	19842176	18584188	19842176	1596773	18245403	93.7	100.0	8.0
87119-Sample_20_S21_R1	Y	15289772	14107182	15289772	5896338	9393434	92.3	100.0	38.6
87119-Sample_21_S22_R1	Y	22209785	20333334	22209785	10178787	12030998	91.6	100.0	45.8
87119-Sample_22_S23_R1	Y	14222750	13167454	14222750	536279	13686471	92.6	100.0	3.8
87119-Sample_23_S2_R1	Y	18051133	17868917	18051133	976446	17074687	99.0	100.0	5.4
87119-Sample_24_S3_R1	Y	29175108	27061980	29175108	3377368	25797740	92.8	100.0	11.6
87119-Sample_25_S4_R1	Y	21955319	21093654	21955319	1946825	20008494	96.1	100.0	8.9
87119-Sample_26_S5_R1	Y	10229839	9900297	10229839	1020967	9208872	96.8	100.0	10.0
87119-Sample_27_S6_R1	Y	9248787	8581398	9248787	1558206	7690581	92.8	100.0	16.8
87119-Sample_28_S7_R1	Y	23977184	23024756	23977184	2083570	21893614	96.0	100.0	8.7
87119-Sample_9_S10_R1	Y	9938720	9238178	9938720	2253601	7685119	93.0	100.0	22.7

Table 3: Preprocessing. General statistics on QC and adapter trimming. **r.total**: Total number of reads, **r.adapt**: reads with adapter trimmed, **r.pass**: trimmed reads passing base quality filter, **r.short**: reads shorter than 18bp threshold, **r.final**: final number of reads for analysis, **pc.adapt**: percent of total reads with adapter trimmed, **pc.pass**: percent of reads passing base quality threshold, **pc.short**: percent of reads removed due to read length.

% latex table generated in R 3.4.3 by xtable 1.8-2 package % Tue May 22 13:06:35 2018

% latex table generated in R 3.4.3 by xtable 1.8-2 package % Tue May 22 13:06:35 2018

Information on Method 2 database versions

```
SeqMap version/date: ..................... 1.0.13
Genomic tRNA database version/date: ....... 29.03.2018 (dd.mm.yyyy)
piRNA cluster database version/date: ...... 29.03.2018 (dd.mm.yyyy)
```

sample	case	r.total	r.unmap	r.onemap	r.ambig	pc.unmap	pc.onemap	pc.ambig
87119-Sample_1_S2_R1	N	6363983	2755361	617601	2991021	43.3	9.7	47.0
87119-Sample_2_S3_R1	N	13581022	2332607	1053234	10195181	17.2	7.8	75.1
87119-Sample_29_S8_R1	N	14779615	9428255	2520074	2831286	63.8	17.1	19.2
87119-Sample_3_S4_R1	N	12017577	4169078	948404	6900095	34.7	7.9	57.4
87119-Sample_30_S9_R1	N	6030569	3560662	1319243	1150664	59.0	21.9	19.1
87119-Sample_4_S5_R1	N	3219060	1786788	633303	798969	55.5	19.7	24.8
87119-Sample_5_S6_R1	N	23736442	5152286	2720082	15864074	21.7	11.5	66.8
87119-Sample_6_S7_R1	N	3904200	1631617	419629	1852954	41.8	10.7	47.5
87119-Sample_7_S8_R1	N	24083598	10406854	2022669	11654075	43.2	8.4	48.4
87119-Sample_8_S9_R1	N	13699647	11192085	947751	1559811	81.7	6.9	11.4
87119-Sample_10_S11_R1	Y	15470169	4698959	1198870	9572340	30.4	7.7	61.9
87119-Sample_11_S12_R1	Y	18551316	3580693	2338564	12632059	19.3	12.6	68.1
87119-Sample_12_S13_R1	Y	15017069	7325602	1833183	5858284	48.8	12.2	39.0
87119-Sample_13_S14_R1	Y	20092825	6025788	4427587	9639450	30.0	22.0	48.0
87119-Sample_14_S15_R1	Y	15630782	6181187	2243315	7206280	39.5	14.4	46.1
87119-Sample_15_S16_R1	Y	14490875	2710319	1324638	10455918	18.7	9.1	72.2
87119-Sample_16_S17_R1	Y	21327104	6605087	9073681	5648336	31.0	42.5	26.5
87119-Sample_17_S18_R1	Y	16514844	6291929	2596605	7626310	38.1	15.7	46.2
87119-Sample_18_S19_R1	Y	15616291	2831331	2386846	10398114	18.1	15.3	66.6
87119-Sample_19_S20_R1	Y	18245403	7669336	2625532	7950535	42.0	14.4	43.6
87119-Sample_20_S21_R1	Y	9393434	4102374	3413026	1878034	43.7	36.3	20.0
87119-Sample_21_S22_R1	Y	12030998	5237631	4158991	2634376	43.5	34.6	21.9
87119-Sample_22_S23_R1	Y	13686471	5010873	1319712	7355886	36.6	9.6	53.7
87119-Sample_23_S2_R1	Y	17074687	7019002	2529727	7525958	41.1	14.8	44.1
87119-Sample_24_S3_R1	Y	25797740	8334680	4091616	13371444	32.3	15.9	51.8
87119-Sample_25_S4_R1	Y	20008494	8002503	1603987	10402004	40.0	8.0	52.0
87119-Sample_26_S5_R1	Y	9208872	3031628	1394710	4782534	32.9	15.1	51.9
87119-Sample_27_S6_R1	Y	7690581	1575490	1599587	4515504	20.5	20.8	58.7
87119-Sample_28_S7_R1	Y	21893614	7969039	1971860	11952715	36.4	9.0	54.6
87119-Sample_9_S10_R1	Y	7685119	3200687	1330232	3154200	41.6	17.3	41.0

Table 4: Alignment statistics using 'bowtie2'. **r.total**: total number of reads for alignment, **r.unmap**: reads not mapped to the GRCh38 human genome, **r.onemap**: reads mapping uniquely to single locus, **r.ambig**: reads with multiple mappings, **pc.unmap**: percent of total reads not mapped, **pc.onemap**: percent of total reads mapping uniquely, **pc.ambig**: percent of reads with multiple mappings.

```
Ensembl version/date: ..................... Release 91
EnsemblGenomes version/date: .............. Release 38
tRF-1 sequence data version/date: ......... 19.12.2017 (dd.mm.yyyy)
tRNA-leader sequence data version/date: ... 19.12.2017 (dd.mm.yyyy)
SILVA rRNA (SSU) database version/date: ... Release 132
SILVA rRNA (LSU) database version/date: ... Release 132
miRBase database version/date: ............ Release 22
```

% latex table generated in R 3.4.3 by xtable 1.8-2 package % Tue May 22 13:06:35 2018

sample	case	r.feature	r.nomap	r.nofeature	r.ambig2	pc.feat
87119-Sample_1_S2_R1	N	1883842	3580693	12487499	599282	16.6
87119-Sample_2_S3_R1	N	64213	5237631	6726990	2164	1.0
87119-Sample_29_S8_R1	N	906547	4102374	4312356	72157	18.5
87119-Sample_3_S4_R1	N	1245637	4169078	4786596	1816266	39.0
87119-Sample_30_S9_R1	N	38918	3560662	2426876	4113	1.7
87119-Sample_4_S5_R1	N	118169	1786788	1300876	13227	9.2
87119-Sample_5_S6_R1	N	3822808	5152286	12721415	2039933	31.5
87119-Sample_6_S7_R1	N	124574	1631617	2088811	59198	8.1
87119-Sample_7_S8_R1	N	2721151	10406854	9408849	1546744	31.2
87119-Sample_8_S9_R1	N	61213	11192085	2442369	3980	2.6
87119-Sample_10_S11_R1	Y	1955044	7325602	3336711	2399712	56.6
87119-Sample_11_S12_R1	Y	1917358	6025788	11204166	945513	20.4
87119-Sample_12_S13_R1	Y	2988956	6181187	3983902	2476737	57.8
87119-Sample_13_S14_R1	Y	1035771	2710319	10337186	407599	12.3
87119-Sample_14_S15_R1	Y	54453	6605087	14654382	13182	0.5
87119-Sample_15_S16_R1	Y	2807474	6291929	5060694	2354747	50.5
87119-Sample_16_S17_R1	Y	2442847	2831331	10148700	193413	20.6
87119-Sample_17_S18_R1	Y	2763370	7669336	5631161	2181536	46.8
87119-Sample_18_S19_R1	Y	143663	2755361	3416534	48425	5.3
87119-Sample_19_S20_R1	Y	548119	4698959	9766876	456215	9.3
87119-Sample_20_S21_R1	Y	1805418	5010873	4438233	2431947	48.8
87119-Sample_21_S22_R1	Y	5458877	7019002	3734248	862560	62.9
87119-Sample_22_S23_R1	Y	2008725	8334680	11121195	4333140	36.3
87119-Sample_23_S2_R1	Y	1945922	8002503	4364371	5695698	63.6
87119-Sample_24_S3_R1	Y	1170291	3031628	4025815	981138	34.8
87119-Sample_25_S4_R1	Y	540490	1575490	5539389	35212	9.4
87119-Sample_26_S5_R1	Y	1746826	7969039	7082064	5095685	49.1
87119-Sample_27_S6_R1	Y	67895	9428255	5272014	11451	1.5
87119-Sample_28_S7_R1	Y	1332497	2332607	9386774	529144	16.6
87119-Sample_9_S10_R1	Y	80010	3200687	4401875	2547	1.8

Table 5: Read count statistics on known miR features, method 1. microRNA features from miRbase, v22, 2018-05. **r.feature**: reads overlapping known features, **r.nomap**: reads not mapping to genome (same as r.unmap in alignment stats), **r.nofeat**: reads not mapping over features, **r.ambig2**: reads counting towards more than one feature, **pc.feat**: percent of mapped reads counting towards a feature.

sample	case	miRNA:hs	miRNA:oth	3povlpncRNA	unk	rRNA:gen	tRNA:gen	other
87119-Sample_1_S2_R1	N	197802	18295	272.2	1096419	725968.5	337626.0	224255.3
87119-Sample_2_S3_R1	N	1864600	13438	63.8	1910189	784285.8	843160.7	714436.1
87119-Sample_29_S8_R1	N	80738	5357	713.2	4003589	300282.8	118993.1	298023.9
87119-Sample_3_S4_R1	N	3069439	16576	85.4	1827045	993223.7	683932.1	267143.2
87119-Sample_30_S9_R1	N	44828	4564	689.1	1975575	207779.5	39366.6	150521.6
87119-Sample_4_S5_R1	N	132296	1905	255.8	795742	73540.9	18744.5	74221.6
87119-Sample_5_S6_R1	N	5868155	25119	119.6	3346266	1553639.0	1355386.4	1258334.0
87119-Sample_6_S7_R1	N	184928	4435	110.1	539638	213043.3	145875.3	92127.6
87119-Sample_7_S8_R1	N	4272957	26548	164.1	2698599	1515541.9	522552.7	780967.1
87119-Sample_8_S9_R1	N	65664	24395	361.3	1363709	894451.1	38511.0	105389.3
87119-Sample_10_S11_R1	Y	1025662	40911	551.3	3858951	3855838.9	935657.0	296073.9
87119-Sample_11_S12_R1	Y	2508341	40852	244.7	4880643	3773356.1	2442214.1	340939.8
87119-Sample_12_S13_R1	Y	4360570	24383	223.1	1718470	625612.3	356610.1	169715.8
87119-Sample_13_S14_R1	Y	2892879	67153	699.6	5657243	2033151.6	1152060.3	376353.5
87119-Sample_14_S15_R1	Y	5472881	16852	176.6	1410805	735565.8	605955.7	188869.0
87119-Sample_15_S16_R1	Y	1479944	47757	240.8	3604377	2779441.3	698701.0	335909.7
87119-Sample_16_S17_R1	Y	70039	4338	7455.7	13384160	143258.9	33839.6	1070898.5
87119-Sample_17_S18_R1	Y	5176052	24406	307.2	2101065	758523.7	505457.4	190584.9
87119-Sample_18_S19_R1	Y	2665786	50517	511.3	5594214	2752601.9	1003827.4	246359.5
87119-Sample_19_S20_R1	Y	4959732	32449	303.5	2462877	991911.7	557383.5	232762.3
87119-Sample_20_S21_R1	Y	970089	6075	1105.4	3745672	202435.2	40243.1	251131.1
87119-Sample_21_S22_R1	Y	66449	4272	1814.8	6057736	150805.2	90881.0	415146.3
87119-Sample_22_S23_R1	Y	4245288	16051	100.7	1933722	1488907.3	282124.7	133914.1
87119-Sample_23_S2_R1	Y	6328899	14931	81.5	453624	333506.9	96725.0	144187.9
87119-Sample_24_S3_R1	Y	6376649	59826	1202.3	5823955	2167186.6	823109.8	473028.7
87119-Sample_25_S4_R1	Y	7649872	24629	209.0	2073009	1333935.8	261081.0	206436.6
87119-Sample_26_S5_R1	Y	2157259	16930	304.1	1301900	323736.7	1132743.3	276893.5
87119-Sample_27_S6_R1	Y	600535	21170	303.9	2738079	1735792.8	568571.5	165170.3
87119-Sample_28_S7_R1	Y	6849865	14962	305.7	2693642	804827.1	815749.6	340050.3
87119-Sample_9_S10_R1	Y	94929	33709	689.8	2401052	1307788.2	228720.8	385821.6

Table 6: Summary statistics of read mapping by method 2. **miRNA:hs**: reads mapped to human microRNA sequences (combination of miRbase and Ensemble transcripts), **miRNA:oth**: reads mapping to other non-human species microRNA, **3povlpncRNA**: normalised reads mapping to 3' overlapping non-coding RNA, **unk**: reads of unknown mapping (non-human or other RNA fragments), **rRNA:gen**: ribosomal RNA of genomic origin (normalised), **tRNA: gen**: transcriptomic RNA of genomic origin (normalised), **other**: other small RNA features (low complexity RNA, mitocondrial ribosomal and transcriptomic fragments, snRNA, snoRNA, scaRNA, ribozyme, lincRNA, macro lncRNA, protein coding RNA, etc.).

HUNTER NEW ENGLAND
NSW HEALTH

7 July 2010

Dr A Enjeti
Haematology Unit
Calvary Mater Newcastle

Dear Dr Enjeti,

Re: Circulating Microparticles to Detect Thrombosis, Bleeding and as a Prognostic Marker in Haematological Malignancies (06/12/13/5.05)

Thank you for submitting a request for an amendment to the above project. This amendment was reviewed by the Hunter New England Human Research Ethics Committee. This Human Research Ethics Committee is constituted and operates in accordance with the National Health and Medical Research Council's *National Statement on Ethical Conduct in Human Research (2007)* (National Statement) and the *CPMP/ICH Note for Guidance on Good Clinical Practice.* Further, this Committee has been accredited by the NSW Department of Health as a lead HREC under the model for single ethical and scientific review.

I am pleased to advise that the Hunter New England Human Research Ethics Committee has granted ethical approval for the following amendment request:

- For the Participant Information Sheet and Consent Form (Version 2 dated 30 June 2010)

Approval from the Hunter New England Human Research Ethics Committee for the above protocol is given for a maximum of **3** years from the date of the approval letter of your initial application, after which a renewal application will be required if the protocol has not been completed. The above protocol is approved until **December 2012.**

The *National Statement on Ethical Conduct in Human Research (2007)* which the Committee is obliged to adhere to, include the requirement that the committee monitors the research protocols it has approved. In order for the Committee to fulfil this function, it requires:

- a report of the progress of the above protocol be submitted at 12 monthly intervals. Your review date is **December 2010.** A proforma for the annual report will be sent two weeks prior to the due date.

- A final report be submitted at the completion of the above protocol, that is after data analysis has been completed and a final report compiled. A proforma for the final report will be sent two weeks prior to the due date.

Hunter New England Human Research Ethics Committee

(Locked Bag No 1)
(New Lambton NSW 2305)
Telephone (02) 49214 950 Facsimile (02) 49214 818
Email:hnehrec@hnehealth.nsw.gov.au
Nicole.gerrand@hnehealth.nsw.gov.au
Michelle.lane@hnehealth.nsw.gov.au
http://www.hnehealth.nsw.gov.au/Human_Research_Ethics

- All variations or amendments to this protocol, including amendments to the Information Sheet and Consent Form, must be forwarded to and approved by the Hunter New England Human Research Ethics Committee prior to their implementation.

- The Principal Investigator will immediately report anything which might warrant review of ethical approval of the project in the specified format, including:
    - any serious or unexpected adverse events
        - Adverse events, however minor, must be recorded as observed by the Investigator or as volunteered by a participant in this protocol. Full details will be documented, whether or not the Investigator or his deputies considers the event to be related to the trial substance or procedure.

        - Serious adverse events that occur during the study or within six months of completion of the trial at your site should be reported to the Professional Officer of the Hunter New England Human Research Ethics Committee as soon as possible and at the latest within 72 hours.

        - Copies of serious adverse event reports from other sites should be sent to the Hunter New England Human Research Ethics Committee for review as soon as possible after being received.

        - Serious adverse events are defined as:

            - Causing death, life threatening or serious disability.
            - Cause or prolong hospitalisation.
            - Overdoses, cancers, congenital abnormalities whether judged to be caused by the investigational agent or new procedure or not.

    - unforeseen events that might affect continued ethical acceptability of the project.

- If for some reason the above protocol does not commence (for example it does not receive funding); is suspended or discontinued, please inform Dr Nicole Gerrand, the Professional Officer of the Hunter New England Human Research Ethics Committee as soon as possible.

The Hunter New England Human Research Ethics Committee also has delegated authority to approve the commencement of this research on behalf of the Hunter New England Area Health Service. This research may therefore commence.

Should you have any queries about your project please contact Dr Nicole Gerrand as per her contact details at the bottom of the page. The Hunter New England Human Research Ethics Committee Terms of Reference, Standard Operating Procedures, membership and standard forms are available from the Hunter New England Area Health Service website:

Internet address: http://www.hnehealth.nsw.gov.au/Human_Research_Ethics

Please quote **06/12/13/5.05** in all correspondence.

Hunter New England Human Research Ethics Committee

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(New Lambton NSW 2305)
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Michelle.lane@hnehealth.nsw.gov.au
http://www.hnehealth.nsw.gov.au/Human_Research_Ethics

The Hunter New England Human Research Ethics Committee wishes you every success in your research.

Yours faithfully

For: Dr M Parsons
Chair
Hunter New England Human Research Ethics Committee

Hunter New England Human Research Ethics Committee

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(New Lambton NSW 2305)
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http://www.hnehealth.nsw.gov.au/Human_Research_Ethics

1 February 2017

Dr M Seldon
Haematology Unit
Calvary Mater Newcastle

Dear Dr Seldon

Re: Circulating Microparticles to Detect Thrombosis, Bleeding and as a Prognostic Marker in Haematological Malignancies (06/12/13/5.05)

Thank you for submitting the renewal application for the above project which was considered by the Hunter New England Human Research Ethics Committee at its executive meeting held on **1 February 2017. Retrospective renewal has been granted from December 2016.** This Human Research Ethics Committee is constituted and operates in accordance with the National Health and Medical Research Council's *National Statement an Ethical Conduct in Human Research 2007* and the *CPMP/ICH Note for Guidance on Good Clinical Practice*.

I am pleased to advise that the Hunter New England Human Research Ethics Committee has granted ongoing ethical approval of the above protocol having confirmed that the project continues to meet the requirements of the *National Statement on Ethical Conduct in Human Research*.

Ethics Approval will be ongoing subject to the following conditions:

- A report of the progress of the above protocol is to be submitted at 12 monthly intervals. Your review date is **December 2017.** A proforma for the annual report will be sent at the beginning of the month of the anniversary of approval.
- All variations or amendments to this protocol, including amendments to the Information Sheet and Consent Form, must be forwarded to and approved by the Hunter New England Human Research Ethics Committee prior to their implementation.
- A final report must be submitted at the completion of the above protocol, that is, after data analysis has been completed and a final report compiled.

Please Note: The committee reserves the right to re-evaluate this decision at any time.

Approval has been granted for this study to continue at the following site:

- **Calvary Mater Newcastle**

The *National Statement an Ethical Conduct in Human Research 2007,* to which the Committee is obliged to adhere, includes the requirement that the Committee monitors the research protocols it has approved. In order for the Committee to fulfil this function, in addition to the conditions of approval set out above, it requires:

Hunter New England Research Ethics & Governance Office
Locked Bag No 1
New Lambton NSW 2305
Telephone: (02) 49214950
Email: HNELHD-HREC@hnehealth.nsw.gov.au
http://www.hnehealth.nsw.gov.au/ethics/Pages/Research-Ethics-and-Governance-Unit.aspx

- The Principal Investigator will immediately report anything which might warrant review of ethical approval of the project in the specified format, including:

    - any serious or unexpected adverse events

        - Adverse events, however minor, must be recorded as observed by the Investigator or as volunteered by a participant in this protocol. Full details will be documented, whether or not the Investigator or his deputies considers the event to be related to the trial substance or procedure.

        - Serious adverse events that occur during the study or within six months of completion of the trial at your site should be reported to the Professional Officer of the Hunter New England Human Research Ethics Committee as soon as possible and at the latest within 72 hours.

        - Copies of serious adverse event reports from other sites should be sent to the Hunter New England Human Research Ethics Committee for review as soon as possible after being received.

        - Serious adverse events are defined as:

            - Causing death, life threatening or serious disability.
            - Cause or prolong hospitalisation.
            - Overdoses, cancers, congenital abnormalities whether judged to be caused by the investigational agent or new procedure or not.
            - Unforeseen events that might affect continued ethical acceptability of the project.

        - If for some reason the above protocol does not commence (for example it does not receive funding); is suspended or discontinued, please inform Dr Nicole Gerrand, the Manager, Research Ethics & Governance Office, as soon as possible.

The Hunter New England Human Research Ethics Committee also has delegated authority to approve the commencement of this research on behalf of the Hunter New England Local Health District. This research may therefore continue.

Should you have any queries about your project please contact Dr Nicole Gerrand as per the contact details at the bottom of the page. The Hunter New England Human Research Ethics Committee Terms of Reference, Standard Operating Procedures, membership and standard forms are available from the Hunter New England Area Health Service website.

Please quote **06/12/13/5.05** in all correspondence.

The Hunter New England Human Research Ethics Committee wishes you every success in your research.

Yours faithfully

For: Ms M Hunter
Chair
Hunter New England Human Research Ethics Committee

Hunter New England Research Ethics & Governance Office
Locked Bag No 1
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Email: HNELHD-HREC@hnehealth.nsw.gov.au
http://www.hnehealth.nsw.gov.au/ethics/Pages/Research-Ethics-and-Governance-Unit.aspx

HUMAN RESEARCH ETHICS COMMITTEE

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

Notification of Expedited Approval

To Chief Investigator or Project Supervisor:	**Dr Michael Seldon**
Cc Co-investigators / Research Students:	**Dr Anoop Enjeti** **Dr Lisa Lincz**
Re Protocol:	**Circulating Microparticles to Detect Thrombosis, Bleeding and as a Prognostic Marker in Haematological Malignancies**
Date:	**15-Oct-2012**
Reference No:	**H-2012-0316**

Thank you for your **Variation** submission to the Human Research Ethics Committee (HREC) seeking approval in relation to a variation to the above protocol.

Variation to:

1. Add Dr Michael Seldon as Principal Investigator
2. Change the role of Dr Anoop Enjeti from Principal Investigator to Student Researcher

- Research Plan, version 2 dated 6.3.2012
- Participant Information Sheet and Consent Form, Calvary Mater Newcastle, version 3 dated 6.3.2012)

Your submission was considered under **Expedited Review of External Approval** review by the Chair/Deputy Chair.

I am pleased to advise that the decision on your submission is **External HREC Approval Noted** effective **09 -Oct-2012**.

The full Committee will be asked to ratify this decision at its next scheduled meeting. A formal *Certificate of Approval* will be available upon request.

Professor Allyson Holbrook
Chair, Human Research Ethics Committee

For communications and enquiries:
Human Research Ethics Administration

Research Services
Research Integrity Unit
HA148, Hunter Building
The University of Newcastle

Callaghan NSW 2308
T +61 2 492 18999
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Human-Ethics@newcastle.edu.au

RIMS website - https://RIMS.newcastle.edu.au/login.asp

Linked University of Newcastle administered funding:

Funding body	Funding project title	First named investigator	Grant Ref

PARTICIPANT INFORMATION SHEET

Study:	Circulating microparticles to predict thrombosis, bleeding and as a prognostic marker in haematological malignancies
Project Code:	MP in Haem malig (short abbreviation)
Name of Investigator:	Michael Seldon
Name of Student Investigator:	Anoop K Enjeti (PhD Student, University of Newcastle)

1. YOUR CONSENT

You are invited to take part in the above research project that is being conducted by Dr.Enjeti at the Calvary Mater Newcastle.

This Participant Information Sheet contains detailed information about the research project. Its purpose is to explain to you as openly and clearly as possible all the procedures involved in this project before you decide whether or not to take part in it. Please read this Participant Information Sheet carefully. Feel free to ask questions about any information in the Information Sheet. Before deciding whether or not to take part, you may wish to discuss the project with a relative or friend or your local health worker. Once you understand what the project is about and if you agree to take part in it, you will be asked to sign the Consent Form. By signing the Consent Form you indicate that you understand the information and that you give your consent to participate in the research project.

You have been asked to participate in this study because you have a condition such as a myeloproliferative disorder, myelodysplasia/leukemia, lymphoma or myeloma which is being treated at the Calvary Mater Newcastle. Patients with these conditions are at an increased risk for developing abnormal clotting (referred to as 'thrombosis') or bleeding during the course of their treatment.

The aim of the study is to evaluate the ability of a new laboratory test to help predict the clotting or bleeding complications as well as prognosis. This study is being undertaking as part of Dr Enjeti's studies for his PhD at the University of Newcastle under the supervision of Dr Michael Seldon.

2. PARTICIPATION IS VOLUNTARY

Participation in any research project is voluntary. If you do not wish to take part you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage. Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with the treating hospital. Before you make your decision, a member of the research team will be available so that you can ask any questions you have about the research project. You can ask for any information you want. Sign the Consent Form only after you have had a chance to ask your questions and have received satisfactory answers.

If you decide to withdraw from this project, please notify a member of the research team before you withdraw. This notice will allow that person or the research supervisor to inform you if there are any health risks or special requirements linked to withdrawing the treatment. If you do withdraw from the study, you have the option of withdrawing all data relating to you.

3. WHAT WILL HAPPEN ON THE STUDY

If you agree, an additional blood sample (approximately 5mls) will be collected while collecting samples during your routine follow up for your disease. This will be required at each of your follow up visits while on the study. As you will be having blood collected as a part of your routine

testing, an extra sample (5 ml) will be collected for research purposes during the process. It is expected that routine follow up will occur at 4-6 week intervals. As a part of the study, samples will be collected at each follow up for your disease. This will mean that 8-12 samples from you will be collected over a 1 year period.

The sample collected from you will be used to evaluate a new laboratory test to help predict bleeding or clotting complications. These results will then be will be correlated with your medical diagnosis, blood counts, treatment, history of bleeding or clotting and any genetic predisposition for clotting in order to evaluate the usefulness of the new test. The sample collected from you will be used for this research project alone and will be discarded according to hospital protocol once the tests have been performed.

Only tests that constitute part of the study will be performed on the samples collected. It is possible that in the future, research studies or new testing procedures may be developed in relation to thrombosis. With your consent we would like to continue to securely store your blood sample at the Mater Hospital (for use only in studies carried out at this hospital) for possible future studies. The consent form attached to the information sheet has a section for you to choose yes or no to the secure storage of their blood samples for possible future studies. Any future studies will be carried out on stored samples only after the approval of the Hunter New England Research ethics Committee.

Study samples that are not being kept for possible future research will be safely destroyed according to the hospital protocol after all final study analysis is complete.

4. TESTS WHILE ON STUDY

While on the study all the blood tests and examinations as may be required for the standard of care for your condition will be performed. Participation is entirely voluntary and only samples from people who give their informed consent will be included in the project. If you choose not to have your sample tested as part of the study, this will not affect any future treatment you may require in any way.

5. POSSIBLE OUTCOMES/POTENTIAL BENEFITS

We cannot guarantee or promise that you will receive any direct benefits from this project.

6. ARE THERE ANY RISKS

The only risk associated with this study is that of venepuncture which would have been performed as part of routine follow up.
The removal of blood, by injecting a needle directly into a vein, is a safe procedure, which is unlikely to cause any problems, but can on occasions cause pain, swelling, bruising or infection at the site of the needle puncture. Dizziness or fainting during venesection are other side effects.

All adverse events will be treated according to current hospital protocols. Blood samples are potentially infectious material and will be the only source of biologically hazardous material in this study. All laboratory and scientific staff are trained in the handling and processing of blood samples. All safety precautions and regulations will be followed during the procedures.

7. OTHER TREATMENT WHILST YOU ARE ON THE STUDY

It is important that you tell your doctor about any treatments or medications you may be taking including non-prescription medications, vitamins or herbal remedies, acupuncture or other alternative procedures and any changes to these during your participation in the study.

8. COMPENSATION IN CASE OF INJURY

Every reasonable precaution will be taken to ensure your safety during the course of the study. If you are injured as a direct result of the effects of the study medication, reasonable medical treatment will be provided to you by the Newcastle Mater Hospital.
You must notify your study doctor immediately of any research-related injury and the nature of the expenses to be covered. If you have any question concerning the availability of medical care

of if you think you have experienced a research-related illness, injury or emergency, you should contact your study doctor. In the event that you suffer an injury as a result of participating in this trial, hospital care and treatment will be provided by the public health service at no extra cost to you.

Every reasonable precaution will be taken to ensure your safety during the course of the study. If you are injured as a direct result of the effects of the study medication, reasonable medical treatment will be provided. Any compensation made necessary by the study will be made according to the Medicines Australia Guidelines on compensation for drug induced injury. Your participation in this study will not affect any other right to compensation that you might have under statute or common law.

9. PRIVACY, CONFIDENTIALITY AND DISCLOSURE OF INFORMATION

The information collected in this study will be used and stored in accordance with the NSW Health Records and Information Privacy Act 2002.

a. Storage

The data from most of your tests will be analysed as part of the study. By consenting to take part in this study, you also consent to the use of the data obtained from your tests in the overall analysis of the study. All information in relation to the trial will be stored on computer disk and/or paper file in locked offices in the relevant department of the hospital. Once studies have been completed records will be retained in a locked storage facility for 15 years and then disposed of appropriately.

b. Collection

It may be necessary for some of your health information to be obtained from other health service providers, for example from another hospital, a private pathology laboratory, a radiographer or radiotherapist, your GP or a consultant. If you decide that you do not want to provide all or part of the information requested it may not be possible for you to participate in this trial.

c. Disclosure

Any information obtained in connection with this research project that can identify you will remain confidential and will only be used for the purposes of this research or the management of your lymphoma. It will only be disclosed with your permission, except as required by law.

In any publication, information will be provided in such a way that you cannot be identified. By signing the attached Consent Form, you authorise release of, or access to, this confidential information to the relevant study personnel and regulatory authorities as noted above.

It is desirable that your family doctor be advised of your decision to participate in this research project. By signing the Consent Form, you agree to your family doctor being notified of your decision to participate in this research project.

d. Access

Under the Statutory Legislation, you have the right to access information that is collected and stored about you. You should contact any of the persons outlined in Section 17 and 18 if you wish to access your information.

10. NEW INFORMATION ARISING DURING THE PROJECT

During the research project, new information about the risks and benefits of the project may become known to the researchers. If this occurs, you will be told about this new information.

This new information may mean that you can no longer participate in this research. If this occurs, the person(s) supervising the research will stop your participation. In all cases, you will be offered all available care to suit your needs and medical condition.

11. RESULTS OF PROJECT

It is usual for a number of years to elapse before definitive results of this type of study are available. These are published in medical journals that are available to the public. You should feel free to ask your doctor about this.

12. WHAT COSTS ARE INVOLVED

There are no costs to you for participating in this study.

13. FURTHER INFORMATION

If you would like more information about the study or if there is any matter about it that concerns you, now or in the future, do not hesitate to contact your treating doctor. You may also contact the Principal Investigator of this study, Dr Anoop K Enjeti. He can be contacted on 02 49211220 or 02 49211216 for the study coordinator for this study. A research nurse, will be available to help you with the information sheet and in coordinating collection of your samples.
The Research Nurse can be contacted on 49211217.

Thank you for considering this invitation.
Dr Anoop K Enjeti
Principal Investigator
Staff Haematologist

14. COMPLAINTS ABOUT THIS RESEARCH

This study has been approved by the Hunter New England Human Research Ethics Committee and will be carried out according to the National Statement on Ethical Conduct in Human Research (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.

The conduct of this study at the Calvary Mater Newcastle site has been authorised by the board of the Calvary Mater Hospital. Any person with concerns or complaints about the conduct of this study may also contact the Research Governance Officer on 02 49214950, and quote reference number**: 06/12/13/5.05**

If you have any concerns about your rights in relation to the registry, please contact:
Dr Nicole Gerrand PhD
Manager
Research Ethics and Governance
Hunter New England Health
Locked Bag 1
NEW LAMBTON NSW 2305
Ph: (02) 4921 4950
Fax: (02) 4921 4818
hnehrec@hnehealth.nsw.gov.au

CONSENT FORM

Study: Circulating microparticles to predict thrombosis , bleeding and as a prognostic marker in haematological malignancies

Project Code: MP in Haem malig (short abbreviation)

Name of Investigator: Michael Seldon

Name of Student Investigator: Anoop K Enjeti (PhD Student, University of Newcastle)

I have been given a Patient Information Sheet which has explained the nature, purpose, duration and likely effects of the study and what I will be expected to do.

The details of the procedure(s) proposed have also been explained to me, including the anticipated length of time it will take, the frequency with which the procedure(s) will be performed, and an indication of any discomfort which may be expected.

I understand that my study doctor and the research nurse/data manager involved in this study will have access to my medical records from all hospitals that I have attended as an outpatient/inpatient, for data collection and documentation during this study.

I understand that the study will be conducted as described in the Information Statement, a copy of which I have retained.

I understand that I may withdraw from the study at any time and I do not have to give a reason for withdrawing and this will not affect my further treatment.

I agree that my General Practitioner will be notified about my participation in this study. ☐

I agree to my samples being stored for any future studies on microparticles/ thrombosis: ☐

I disagree to my samples being stored for any future studies on microparticles / thrombosis ☐

Last name of patient:.....……. First Name:.....
(Please print) (Please print)

Signature of patient:.....…… Date:.....

(signature of witness only required if signing on behalf of the participant)

Last name of Witness: .....……. First Name:.....
(Please print) (Please print)

I have discussed this clinical study with the patient and/or his or her authorized representative. I believe that I have fully informed the participant of the nature of this study and its possible benefits and risks, and I believe the participant understood this explanation.

Signature of Investigator/Research Member: ……

Please Print Name:…… Date...……

UON Graduate Research

Co-authorship declaration

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

TITLE AND CITATION	Enjeti AK, Ariyarajah A, D'Crus A, Seldon M, Lincz LF. Correlative analysis of nanoparticle tracking, flow cytometric and functional measurements for circulating microvesicles in normal subjects. *Thromb Res*. 2016;145:18-23.

AUTHOR POSITION	AUTHOR	SPECIFICS OF CONTRIBUTION TO RESEARCH REPORTED AND WRITING OF THE FINAL PAPER	% CONTRIBUTION TO PAPER	SIGNATURE
1	Anoop K Enjeti	Concept, literature review, experimental work, ethics manuscript writing	40	
2	Anita Ariyarajah	Experimental work	20	Anita Ariyarajah (Apr 10, 2018)
3	Angel D'Crus	Experimental work	15	Angel D'Crus (Apr 10, 2018)
4	Michael Seldon	Supervision and manuscript review	10	M Seldon (Aug 27, 2018)
5	Lisa F Lincz	Supervision, analysis and manuscript review	15	

Prof Derek Laver
A/Assistant Dean Research Training (FHEAM)
7 September 2018

UON Graduate Research

Co-authorship declaration

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

TITLE AND CITATION	Enjeti AK, Ariyarajah A, Warwick E, Seldon M, Lincz LF (2017) Challenges in Analysis of Circulating Extracellular Vesicles in Human Plasma Using Nanotracking and Tunable Resistive Pulse Sensing. J Nanomed Nanotechnol 8: 468.

AUTHOR POSITION	AUTHOR	SPECIFICS OF CONTRIBUTION TO RESEARCH REPORTED AND WRITING OF THE FINAL PAPER	% CONTRIBUTION TO PAPER	SIGNATURE
1	Anoop K Enjeti	Concept, literature review, experimental work, ethics manuscript writing	40	
2	Anita Ariyarajah	Experimental work	10	Anita Ariyarajah (Apr 11, 2018)
3	Eleanor Warwick	Experimental work	25	
4	Michael Seldon	Supervision, equipment access and manuscript review	10	M Seldon (Aug 27, 2018)
5	Lisa F Lincz	Supervision, analysis and manuscrip review	15	

Signature: Eleanor Warwick (Apr 30, 2018)

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TITLE AND CITATION	Enjeti AK, Ariyarajah A, D'Crus A, Seldon M, Lincz LF. Circulating microvesicle number, function and small RNA content vary with age, gender, smoking status, lipid and hormone profiles. *Thromb Res.* 2017;156:65-72.

AUTHOR POSITION	AUTHOR	SPECIFICS OF CONTRIBUTION TO RESEARCH REPORTED AND WRITING OF THE FINAL PAPER	% CONTRIBUTION TO PAPER	SIGNATURE
1	Anoop K Enjeti	Concept, literature review, experimental work, ethics manuscript writing	40	
2	Anita Ariyarajah	Experimental work	20	Anita Ariyarajah (Aug 29, 2018)
3	Angel D'Crus	Experimental work	15	Angel D'Crus (Aug 29, 2018)
4	Michael Seldon	Supervision, equipment access and manuscript review	10	M Seldon (Aug 29, 2018)
5	Lisa F Lincz	Supervision, analysis and manuscript review	15	

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Co-authorship declaration

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

TITLE AND CITATION	Anoop K. Enjeti and Michael Seldon (2012). Microparticles: Role in Haemostasis and Venous Thromboembolism, Pathophysiology and Clinical Aspects of Venous Thromboembolism in Neonates, Renal Disease and Cancer Patients, Dr. Mohamed A. Abdelaal (Ed.), InTech, DOI: 10.5772/32080. Available from: https://www.intechopen.com/books/pathophysiology-and-clinical-aspects-of-venous-thromboembolism-in-neonates-renal-disease-and-cancer-patients/microparticles-role-in-haemostasis-and-venous-thromboembolism

AUTHOR POSITION	AUTHOR	SPECIFICS OF CONTRIBUTION TO RESEARCH REPORTED AND WRITING OF THE FINAL PAPER	% CONTRIBUTION TO PAPER	SIGNATURE
1	Anoop K Enjeti	Concept, literature review, manuscript writing	80	
2	Michael Seldon	Supervision and manuscript review	20	M Seldon (Aug 27, 2018)

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07 September 2018

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Co-authorship declaration

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

TITLE AND CITATION	Enjeti AK, Lincz LF, Scorgie FE, Seldon M. Circulating microparticles are elevated in carriers of factor V Leiden. *Thromb Res*. 2010;126(3):250-253.

AUTHOR POSITION	AUTHOR	SPECIFICS OF CONTRIBUTION TO RESEARCH REPORTED AND WRITING OF THE FINAL PAPER	% CONTRIBUTION TO PAPER	SIGNATURE
1	Anoop K Enjeti	Concept, literature review, experimental work,ethics manuscript writing	50	
2	Lisa F Lincz	Supervision, analysis and manuscript review	20	
3	Fiona Scorgie	Experimental work	20	FE Scorgie (Aug 9, 2018)
4	Michael Seldon	Supervision, access to samples and manuscript review	10	M Seldon (Aug 27, 2018)

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7 September 2018

UON Graduate Research

Co-authorship declaration

THE UNIVERSITY OF NEWCASTLE AUSTRALIA

TITLE AND CITATION	**Circulating Microvesicles in Snakebite Patients with Microangiopathy** **Manuscript submitted**

AUTHOR POSITION	AUTHOR	SPECIFICS OF CONTRIBUTION TO RESEARCH REPORTED AND WRITING OF THE FINAL PAPER	% CONTRIBUTION TO PAPER	SIGNATURE
1	Anoop K Enjeti	Concept, experimental work, results and manuscript	40	
2	Lisa Lincz	Supervision, analysis and manuscript review	20	M Seldon (Aug 27, 2018)
3	Michael Seldon	Supervision, analysis and manuscript review	10	
4	Geoff Isbister	Concept, access to patient cohort, ethics, supervision and manuscript review	30	

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7 September 2018

UON Graduate Research

Co-authorship declaration

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TITLE AND CITATION	**Circulating microvesicles are less procoagulant but carry different miRNA cargo in Myelodysplasia** **Manuscript in submission**

AUTHOR POSITION	AUTHOR	SPECIFICS OF CONTRIBUTION TO RESEARCH REPORTED AND WRITING OF THE FINAL PAPER	% CONTRIBUTION TO PAPER	SIGNATURE
1	Anoop K Enjeti	Concept, experimental work, Results and manuscript	40	
2	Anita Ariyarajah	Experimental work	20	Anita Ariyarajah (Aug 6, 2018)
3	Angel D'Crus	Experimental work	10	Angel D'Crus (Aug 7, 2018)
4	Carlos Riveros	Bioinformatic analysis	5	
5	Michael Seldon	Supervision, ethics and manuscript review	10	M Seldon (Aug 27, 2018)
6	Lisa F Lincz	Supervision, analysis and manuscript review	15	

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