Circulating Cell-Derived Microparticles – Potential Markers of Cardiovascular Risk

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Abstract

Circulating cell-derived microparticles, released from cells during activation and apoptosis, are involved in inflammation, coagulation and endothelial dysfunction, all important processes in the development of cardiovascular disease. This project aimed to adapt and validate a flow cytometric assay to measure microparticles derived from various cell types, and to utilise this assay for the investigation of microparticles in healthy individuals and patients with cardiovascular-associated diseases.

A lack of standardisation of pre-analytical variables has impeded the study of microparticles. Pre-analytical variables were analysed, and small changes in methodology were found to have a large impact on microparticle levels detected. Functional microparticle assays were also investigated, and results from these assays were found to be significantly associated with the quantitative results from the flow cytometry assay.

Healthy individuals were recruited to establish a normal range. Interestingly, in healthy individuals, hypoxia induced by exposure to moderate altitude, was shown to cause a decrease in procoagulant, platelet-derived and red blood cell-derived microparticles.

Obstructive sleep apnoea is a common syndrome, associated with an increased risk of cardiovascular disease. Microparticle levels were determined in two randomised controlled trials, investigating the impact of therapy in these patients. Initiation of treatment for six months in minimally symptomatic patients led to a decrease in procoagulant microparticles. Withdrawal of treatment for two weeks in moderate/severe patients led to an increase in endothelial-derived, granulocyte-derived and monocyte-derived microparticles.

Finally circulating microparticles were investigated in patients with cardiovascular-associated conditions. Patients undergoing a dobutamine stress echocardiogram (DSE), for the identification of coronary artery disease, were studied. Patients with a negative DSE exhibited an increase in procoagulant, platelet-derived, endothelial-derived and red blood cell-derived microparticles during stress testing, which was not evident in patients with a positive DSE, suggesting that microparticles provide additional diagnostic value in this setting.

In the rapidly developing field of microparticle analysis, the flow cytometric assay described in this thesis, is reproducible, flexible and correlates well with functional microparticle assays. It has also been shown to provide novel and potentially clinically relevant results in a variety of clinical conditions associated with cardiovascular disease.

List of Publications and Presentations

Publications

1) **Ayers L**, Kohler M, Harrison P, Sargent I, Dragovic R, Schaap M, Nieuwland R, Brooks S, Ferry B. Measurement of circulating cell-derived microparticles by flow cytometry: Sources of variability within the assay. *Thrombosis Research*. 2011 Apr; 127(4):370-7

2) Kohler M, Stoewhas AC, **Ayers L**, Senn O, Bloch KE, Russi EW, Stradling JR. The effects of CPAP therapy withdrawal in patients with obstructive sleep apnea: a randomised controlled trial. *Am J Respir Crit Care Med.* 2011 Nov; 184(10):1192-9

3) **Ayers L**, Stoewhas AC, Ferry B, Stradling JR, Kohler M. Elevated levels of endothelial cell- derived microparticles following withdrawal of CPAP in patients with OSA: a randomized controlled trial. *Respiration*. Accepted, awaiting publication.

4) Stoewhas A, Latshang T, Cascio C, Lautwein S, Stadelmann K, Tesler N, **Ayers L**, Berneis K, Gerber P, Huber R, Achermann P, Bloch K, Kohler M. Effects of acute exposure to moderate altitude on vascular function, metabolism and systemic inflammation. *Chest*. Submitted.

Presentations

1) **Ayers L** *et al*. Impact of methodological variables on the levels of circulating cell-derived microparticles – A biomarker of inflammation. Poster Presentation at 14th International Congress of Immunology, Kobe, Japan (2010).

2) **Ayers L** *et al*. Elevated levels of endothelial microparticles following withdrawal of CPAP in OSA patients: A randomized controlled trial. Poster Presentation at Exosomes and Microvesicles Conference, Orlando, USA (2011).

3) **Ayers L**. Microparticles in Obstructive Sleep Apnoea – A Marker of Heart Disease Risk. Poster Presentation at Oxford Brookes University Symposium, Oxford, UK (2012).

4) **Ayers L**. Measurement of Circulating Cell-Derived Microparticles as Markers of Cardiovascular Risk. Talk at the 1st Oxford Microvesicle and Exosome Group Meeting, Oxford, UK (2012).

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List of Abbreviations

AFM – Atomic Force Microscopy

AHI – Apnoea/Hypopnoea Index

ANCA – Anti-Neutrophil Cytoplasmic Antibodies

APC – Allophycocyanin

BMI – Body Mass Index

BP – Blood Pressure

CAD – Coronary Artery Disease

CD – Cluster of Differentiation

CPAP – Continuous Positive Airway Pressure

CRP – C-Reactive Protein

CVD – Cardiovascular Disease

DLS – Dynamic Light Scattering

DSE – Dobutamine Stress Echocardiogram

ECG – Electrocardiogram

ELISA – Enzyme-Linked Immunosorbant Assay

EM – Electron Microscopy

EMPs – Endothelial Microparticles

ESS – Epworth Sleepiness Score

ETP – Endogenous Thrombin Potential

FITC – Fluoresceinisothiocyanate

FMO – Fluorescence Minus One

fMLP – Formyl-methionine-leucine-phenylalanine

FSc – Forward Scatter

HBSS – Hanks Balanced Saline Solution

HDL – High Density Lipoprotein

HPA – Health Protection Agency

HUVECs – Human Umbilical Cord Vein Endothelial Cell

ICAM-1 – Intracellular Adhesion Molecule 1

IL – Interleukin

IQR – Inter Quartile Range

JAK – Janus Kinase

LDL – Low Density Lipoprotein

LMPs – Leucocyte Microparticles

LPS – Lipopolysaccharide

MOSAIC - Multicentre Obstructive Sleep Apnoea Interventional Cardiovascular Trial

MPs – Microparticles

NO – Nitric Oxide

NTA – Nanoparticle Tracking Analysis

ODI – Oxygen Desaturation Index

OSA – Obstructive Sleep Apnoea

PBS – Phosphate-Buffered Saline

PBS-Ca – PBC-Calcium

PE – Phycoerythrin

PE-Cy5 – Phycoerythrin-Cy5

PerCP – Peridinin Chlorophyll Protein Complex

PHA – Phytohemagglutinin

PMA – Phorbol 12-Myristate 13-Acetate

PMPs – Platelet Microparticles

PPL – Phospholipid

PPP – Platelet-Poor-Plasma

PRP – Platelet Rich Plasma

PS – Phosphatidylserine

RANTES – Regulated upon Activation, Normal T-cell Expressed, and Secreted

RBC MPs – Red Blood Cell-Derived Microparticles

ROCK 1 – Rho-associated coiled-coil containing protein kinase 1

RT – Room Temperature

SMC – Smooth Muscle Cells

SSc – Side Scatter

STAT – Signal Transducer and Activator of Transcription

TGF-β – Transforming Growth Factor-β

TNF – Tumour Necrosis Factor

VLA4 – Very Late Antigen 4

vWF – Von Willebrand Factor

Chapter 1 – Introduction

1.1. Microparticles

1.1.1. Microparticle Definition

Microparticles (MPs) are sub-cellular membrane vesicles, released from cells during activation and apoptosis, and from activated platelets. They range in size from 0.1 μm to 1.0 μm (Baron *et al.*, 2011). They 'bud off' from the plasma membrane of the parent cell, and therefore express many of the surface markers of the cells they originate from (Andrews and Berndt, 2008). These surface markers make it possible to identify subtypes of MPs in the blood, which can be derived from circulating cells such as leucocytes, platelets and erythrocytes (Nomura *et al.*, 2008), as well as tissue sources such as endothelial, tumours and placental tissues (Orozco and Lewis, 2010).

MPs were first reported in 1946, when Chargaff and West noted that a factor accelerated thrombin generation in platelet-free-plasma (Chargaff and West, 1946). Over 20 years later, a coagulant material in particulate form capable of generating thrombin, was identified by electron microscopy in normal plasma, and was labelled as 'platelet dust'(Wolf, 1967).

1.1.2. Microparticle Formation

MP release from cells and platelets can be instigated by a number of triggers, including cytokines, such as tumour necrosis factor (TNF) or interleukin 1 (IL-1), thrombin, calcium ionophore, and by high shear stress (VanWijk *et al.*, 2003).

MPs can be generated by two mechanisms, cell activation and apoptosis. In cell activation, agonists are detected by a cell receptor causing cytosolic calcium to increase (Meziani *et al.*, 2008). This leads to activation of kinases and calpain, inhibition of phosphatases, and talin breakdown. These mechanisms cause disruption of the membrane cytoskeleton and subsequent budding of the membrane, with formation of MPs (VanWijk *et al.*, 2003), as illustrated in Figure 1.1.

In apoptosis, Rho-associated kinase (ROCK 1) is activated by caspase 3 (Coleman *et al.*, 2001), causing cell contraction, redistribution of fragmented DNA and membrane blebbing. MP release tends to occur late in the death process and may occur at the same time as the formation of apoptotic bodies (Ardoin *et al.*, 2007). MPs formed by apoptosis differ from those formed by cell activation in size, in lipid and protein composition, and in their surface markers (VanWijk *et al.*, 2003) (Figure 1.1).

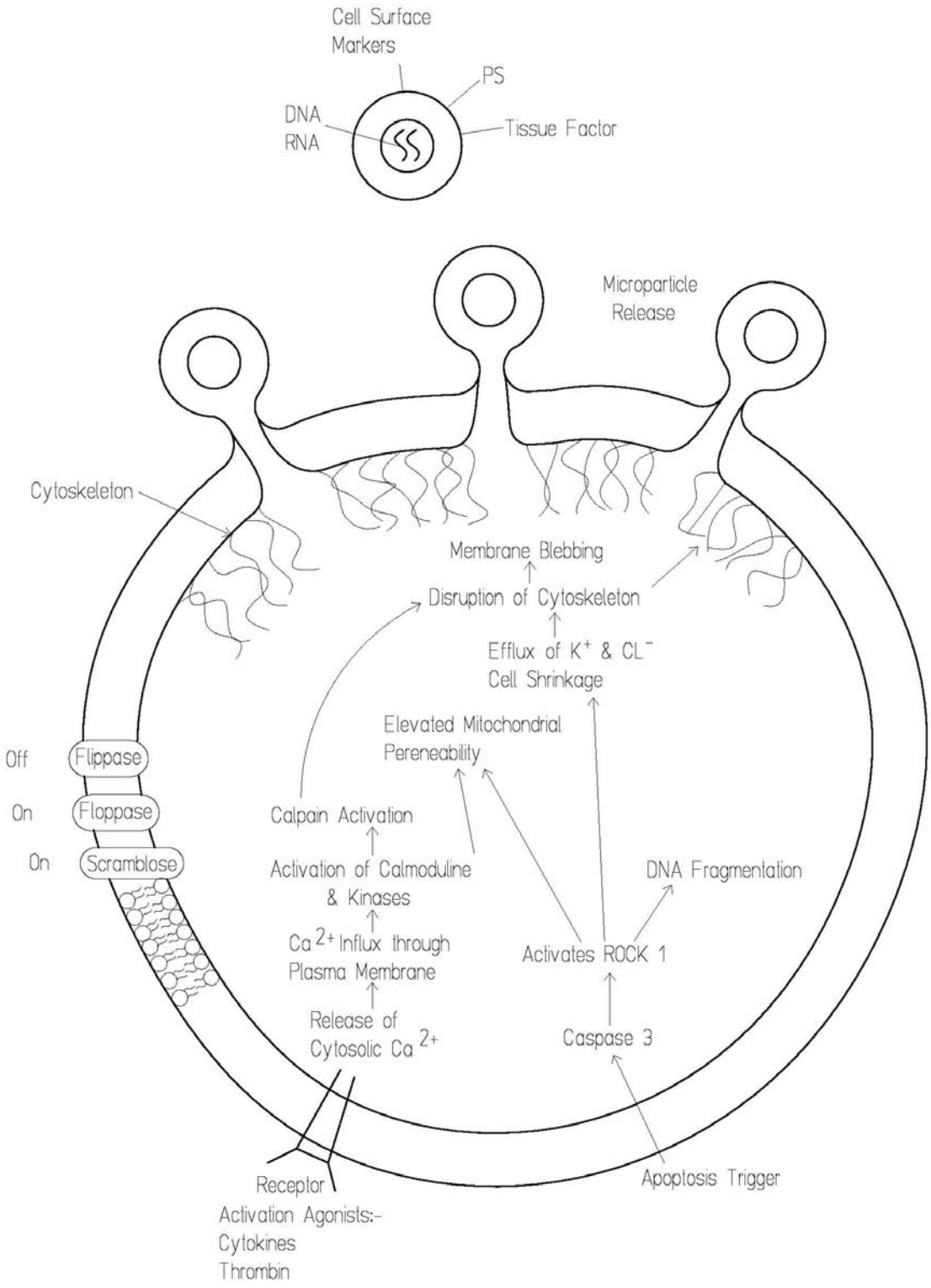

Figure 1.1 Process of microparticle formation during cell activation and apoptosis. Both pathways ultimately lead to disruption of the cytoskeleton and membrane blebbing.

In resting cells, a membrane lipid asymmetry is present, with phosphatidylserine (PS) being segregated to the inner leaflet, and phosphatidylcholine and sphingomyelin being sequestered to the external leaflet. Both cell activation and apoptosis involve a reorganization of the lipid asymmetry of the plasma membrane. Membrane phospholipid asymmetry is controlled by a complex enzyme balance, involving flippase, floppase and

scramblase (Puddu *et al.*, 2010). Flippase specifically and rapidly moves PS from the outer to the inner leaflet. Floppase catalyses the movement of phospholipids from the inner to outer leaflets, and scramblase promotes non-specific redistribution of phospholipids (Morel *et al.*, 2011b). In the absence of stimuli, only flippase is active, maintaining PS on the inner leaflet. The increase in cytosolic calcium that occurs in activation and apoptosis, inhibits flippase, but activates floppase and scramblase (Beyer and Pisetsky, 2010). This leads to the exposure of PS on the outer surface. When the cytoskeleton is no longer able to counteract the surface tension, MP shedding takes place (Freyssinet, 2003).

1.1.3. Distinguishing Microparticles from Other Particles

The other major vesicles released from cells are apoptotic bodies, exosomes and exosome-like vesicles, each with distinguishing features from MPs (Table 1.1).

Apoptotic bodies are larger (>1 μm) and they make up the condensed remnants of shrinking apoptotic cells. Like MPs, they externalise PS, however unlike MPs, they have a permeable membrane allowing staining with propidium iodide (Hristov *et al.*, 2004).

Exosomes are smaller particles (0.05-0.1 μm), formed by inward budding of multivesicular bodies, which are released by exocytosis. They may also be released directly from the plasma membrane. They are differentiated from MPs by their expression of Lamp1 and tetraspanins (CD9, CD63), and their reduced externalisation of PS (Dignat-George and Boulanger, 2011). They only contain cytosolic components and lack organelle proteins (Beyer and Pisetsky, 2010).

Exosome-like vesicles appear to be produced in the same way as exosomes. However, they can be distinguished from exosomes based on their size and sedimentation properties (Gyorgy *et al.*, 2011). They can be distinguished from MPs by their smaller size.

Table 1.1 – Features of Microparticles and Other Particles

Vesicle	Cellular Origin	Size	Shape	Markers	Lipids	References
Microparticles	Plasma membrane	0.1 μm – 1 μm	Irregular	Annexin V+ Cell Specific Markers	Externalise PS Lipid Rafts	(Dignat-George and Boulanger, 2011)
Apoptotic Bodies	Whole Cell	>1 μm	Irregular	Stains with PI, Annexin V+	Externalise PS	(Gyorgy *et al.*, 2011; Hristov *et al.*, 2004)
Exosomes	Internal Multivesicular Bodies	0.05 μm – 0.10 μm	Cup shape	Tetraspanins (CD63, CD9) LAMP 1	Lipid Rafts	(Gyorgy *et al.*, 2011; Thery *et al.*, 2009)
Exosome-Like Vesicles	Internal	0.02 μm – 0.05 μm	Irregular	TNF Receptor 1	No lipid rafts	(Hawari *et al.*, 2004; Thery *et al.*, 2009)

1.1.4. Other Nomenclature for Microparticles

As well as describing cellular vesicles, the term microparticle is commonly used to refer to biopolymer particles, used in drug delivery. As the word particle suggests a particulate or solid structure, it has been suggested that it would be more appropriate to refer to them as microvesicles. This terminology has become more widely used, as has the abbreviation MV (Thery *et al.*, 2009). In some publications, microparticles have been termed ectosomes (Sadallah *et al.*, 2011), because they are released by ectocytosis. For the purposes of this thesis, the term microparticle is used throughout, along with the abbreviation of MP.

1.1.5. Microparticles Subtypes

1.1.5.1. Procoagulant Microparticles

The majority of circulating MPs are procoagulant and expose PS. Procoagulant MPs can be released from any cell, although most circulating procoagulant MPs in healthy individuals are platelet-derived (Berckmans *et al.*, 2001). Procoagulant MPs have been found to be elevated in many conditions including acute coronary syndromes (Mallat *et al.*, 2000), obstructive sleep apnoea (Ayers *et al.*, 2009) and end stage renal failure (Amabile *et al.*, 2005) (Table 1.2).

Annexin V is a phospholipid-binding protein that has a high affinity for PS, in the presence of calcium (Gerke and Moss, 2002). Annexin V binds to procoagulant MPs due to their exposure of PS. It was originally thought that all MPs were annexin V+, and many assays only detect annexin V+ MPs. MPs produced by apoptosis and activation both express PS, although annexin V+ binding may be more evident following apoptosis (Jimenez *et al.*, 2003b).

It is now known that not all MPs are annexin V+ (Boulanger *et al.*, 2006). One possible explanation is that the PS exposed on these MPs is already engaged in other molecular interactions, preventing it from binding annexin V. Another explanation is that some vesicles do not express PS. These MPs may be produced by a similar mechanism described above, but without the loss of membrane asymmetry (Leroyer *et al.*, 2008b).

Annexin V is not the only marker for PS. Lactadherin is a 45 kDa glycoprotein secreted by macrophages. It also binds to PS on apoptotic cells and MPs, and acts as an opsonin, resulting in clearance by macrophages (Dasgupta *et al.*, 2008). Lactadherin preferentially binds to highly curved membranes (Dasgupta *et al.*, 2006), making it a useful marker for MPs (Table 1.3).

1.1.5.2. Platelet-Derived Microparticles

Platelet-derived MPs (PMPs) make up the majority of circulating MPs, in healthy individuals (Berckmans *et al.*, 2001). They are elevated in diabetes (Nomura *et al.*, 1995)

and acute vasculitis (Daniel *et al.*, 2006). PMPs have also been found to be elevated in immune thrombocytopenic purpura, where they are involved in the coagulation and thrombotic complications (Jy *et al.*, 1992). In preeclamptic pregnancies, PMP levels have been shown to be correlated with platelet counts (Lok *et al.*, 2008). PMPs can play a role in the early stages of atherosclerosis, by contributing to thrombosis, enhancing leucocyte adhesion to the endothelium, and promoting inflammation.

Platelets and PMPs share glycoprotein receptors including platelet-endothelial cell adhesion molecule (PECAM, CD31) and integrin αIIbβ3 (CD41/CD61). PMPs generated by platelet activation express activation markers such as P-selectin (CD62P) (Table 1.3). It has been suggested that the majority of CD41+ PMPs are actually derived from megakaryocytes, rather than from platelets, potentially explaining why most do not express CD62P (Flaumenhaft *et al.*, 2009).

1.1.5.3. Endothelial-Derived Microparticles

In healthy individuals endothelial cell-derived MPs (EMPs) are present at much lower numbers in the circulation than PMPs. Levels of EMPs are elevated in several diseases, including acute coronary syndrome (Mallat *et al.*, 2000) and pre-eclampsia (Gonzalez-Quintero *et al.*, 2003), which are inflammatory conditions, suggesting that EMPs play a role in inflammation.

EMPs may also be useful as a marker of disease severity. They are positively correlated with the Birmingham vasculitis activity score in systemic vasculitis (Brogan *et al.*, 2004). In end stage renal failure, EMPs exclusively correlated with *in vivo* markers of endothelial dysfunction (Amabile *et al.*, 2005).

EMPs express PECAM (CD31), VE-Cadherin (CD144), MCAM (CD146), VCAM-1 (CD106), Endoglin (CD105) and E-Selectin (CD62E). CD62E+ and CD106+ EMPs are believed to reflect endothelial cell activation, whereas EMPs with other markers may indicate endothelial cell apoptosis (Jimenez *et al.*, 2003b). With the exception of CD62E and CD144, most of these markers are not exclusive for EMPs. CD31 is expressed on platelets and some leucocyte subsets, and CD146 is expressed on activated T-cells (Dignat-George and Boulanger, 2011) (Table 1.3).

1.1.5.4. Leucocyte-Derived Microparticles

Leucocyte-derived MPs (LMPs) can originate from lymphocytes, granulocytes or monocytes (Angelillo-Scherrer, 2012). They are present at a very low level in the circulation of healthy individuals. They have been found to be elevated in preeclampsia (Lok *et al.*, 2009). They are often increased at the site of inflammation, such as in atherosclerotic plaques (Leroyer *et al.*, 2007) and the synovial fluid of rheumatoid arthritis patients (Berckmans *et al.*, 2002).

Studies have demonstrated that LMPs impair endothelial cell function. *In vitro*, LMPs significantly reduce human umbilical cord vein endothelial cell (HUVEC) survival and proliferation (Yang *et al.*, 2008). LMPs can stimulate endothelial cells (Mesri and Altieri, 1999) and impair endothelium-dependent vasodilation (Martinez *et al.*, 2005).

The main marker used to distinguish total LMPs is the leucocyte common antigen CD45 (Amabile *et al.*, 2008), which identifies MPs derived from lymphocytes, granulocytes and monocytes. Other, more specific markers can be used to identify sub-populations of LMPs (Table 1.3). LMPs from specific cells are discussed below.

1.1.5.5. Granulocyte-Derived Microparticles

Fewer studies have addressed the specific role of granulocyte-derived MPs in disease. Granulocyte-derived MPs have been found to be increased in patients with obstructive sleep apnoea (OSA) (Priou *et al.*, 2010) and in women with a preeclamptic pregnancy (Lok *et al.*, 2009) (Table 1.2). Neutrophils are the most abundant granulocyte in the circulation, and are responsible for the majority of granulocyte-derived MPs.

In vitro, neutrophil-derived MPs can induce the expression of Intracellular Adhesion Molecule 1 (ICAM1) and release of proinflammatory cytokines from endothelial cells, suggesting a role in the pathogenesis of disease (Hong *et al.*, 2012). However, it appears that neutrophil-derived MPs also have anti-inflammatory properties. Neutrophil-derived MPs produced early during neutrophil activation can increase the secretion of Transforming Growth Factor-β (TGF-β) from macrophages, a potent inhibitor of macrophage activation (Gasser and Schifferli, 2004). Neutrophil-derived MPs also contain functionally active annexin 1, which has anti-inflammatory properties (Dalli *et al.*, 2008).

Granulocyte-derived MPs can be identified by their expression of carcinoembryonic antigen-related cell adhesion molecule 8 (CD66B) (George, 2008), an activation marker for human granulocytes (Table 1.3).

1.1.5.6. Monocyte-Derived Microparticles

Monocyte-derived MPs are elevated during preeclamptic pregnancies versus healthy pregnancies (Lok *et al.*, 2009) and in Type II diabetes (Nomura *et al.*, 2009) (Table 1.2). They have the highest procoagulant activity of all MPs, because they express tissue factor (CD142) (Mackman, 2009), a protein required for the initiation of thrombin generation. Monocyte-derived MPs have been found to support faster fibrin formation and have higher clot stability than PMPs (Aleman *et al.*, 2011), suggesting that they play an important role in thrombosis. They have been shown to cause activation of the endothelium, by delivering IL-1β (Wang *et al.*, 2011). Monocyte-derived MPs express CD14, a lipopolysaccharide receptor (Shet *et al.*, 2003) (Table 1.3).

1.1.5.7. T Cell-Derived Microparticles

T cell-derived MPs have been examined in a limited number of studies. They were found to be elevated in active hepatitis C, compared to patients with mild hepatitis C. These MPs were shown to fuse with the hepatic stellate cells, the major cells involved in excess matrix deposition and in liver fibrosis (Kornek *et al.*, 2011). They are also elevated in polymyositis and dermatomyositis patients compared to healthy controls (Baka *et al.*, 2010) (Table 1.2). T cell-derived MPs can be identified by their expression of CD3, CD4 and CD8 surface markers (Table 1.3).

1.1.5.8. Red Blood Cell-Derived Microparticles

Red blood cell derived MPs (RBC MPs) are detectable in the plasma of healthy individuals. RBC MPs are elevated in sickle cell disease patients. In fact, they constitute the majority of circulating MPs in patients with this disease (Shet *et al.*, 2003). RBC MPs are also elevated in nephrotic syndrome, where they may be in part responsible for the thrombophilic susceptibility of these patients (Gao *et al.*, 2012). Elevated levels of RBC MPs can be detected in allogeneic hematopoietic stem-cell transplant patients who develop graft-versus-host disease (Rank *et al.*, 2011b).

RBC MPs can be detected using a monoclonal antibody against glycophorin A (CD235a), a membrane spanning protein present on the surface of RBCs (Shet *et al.*, 2003; Xiong *et al.*, 2012) (Table 1.3).

Table 1.2. Microparticle Subtypes in Disease

MP Subtype	Diseases in which Elevated MP Levels are Found	References
Procogulant MPs	Acute Coronary Syndromes	(Mallat *et al.*, 2000)
	Obstructive Sleep Apnoea	(Ayers *et al.*, 2009)
	End Stage Renal Failure	(Amabile *et al.*, 2005)
Platelet-Derived MPs	Diabetes Mellitus	(Nomura *et al.*, 1995)
	Severe Hypertension	(Preston *et al.*, 2003)
	Obstructive Sleep Apnoea	(Ayers *et al.*, 2009)
	Meningococcal Sepsis	(Nieuwland *et al.*, 2000)
	Active Vasculitis	(Daniel *et al.*, 2006)
Endothelial-Derived MPs	Acute Coronary Syndromes	(Mallat *et al.*, 2000)
	Severe Hypertension	(Preston *et al.*, 2003)
	End Stage Renal Failure	(Amabile *et al.*, 2005)
	Type II Diabetes	(Nomura *et al.*, 2009)
Leucocyte-Derived MPs	Subclinical Atherosclerosis	(Chironi *et al.*, 2006)
	Obstructive Sleep Apnoea	(Ayers *et al.*, 2009)
	Preeclampsia	(Lok *et al.*, 2009)
Granolocyte-Derived MPs	Meningococcal Sepsis	(Nieuwland *et al.*, 2000)
	Obstructive Sleep Apnoea	(Priou *et al.*, 2010)
	Preeclampsia	(VanWijk *et al.*, 2002)
Monocyte-Derived MPs	Preeclampsia	(Lok *et al.*, 2009)
	Type II Diabetes	(Nomura *et al.*, 2009)
T-cell Derived MPs	Polymyositis/Dermatomyositis	(Baka *et al.*, 2010)
	Active Hepatitis C	(Kornek *et al.*, 2011)
	Preeclampsia	(VanWijk *et al.*, 2002)
RBC Derived MPs	Sickle Cell Disease	(Shet *et al.*, 2003)
	Nephrotic Syndrome	(Gao *et al.*, 2012)
	End Stage Renal Failure	(Amabile *et al.*, 2005)

Table 1.3 Microparticle Subtypes and their Surface Markers (* Activation Marker)

MP Subtype	Surface Markers	Alternative Name	Reference
Procogulant MPs	Annexin V	Annexin V	(Dachary-Prigent *et al.*, 1993)
	Lactadherin	Lactadherin	(Hou *et al.*, 2011)
Platelet-Derived MPs	CD31	Platelet-endothelial cell adhesion molecule (PECAM)	(Amabile *et al.*, 2012)
	CD41	Platelet glycoprotein II	(Amabile *et al.*, 2008)
	CD42a	Glycoprotein IX	(Connor *et al.*, 2010)
	CD42b	Glycoprotein Ib	(Connor *et al.*, 2010)
	CD61	Glycoprotein IIIα	(Connor *et al.*, 2010)
	CD62P*	P-selectin	(Flaumenhaft *et al.*, 2009)
Endothelial-Derived MPs	CD31	Platelet-endothelial cell adhesion molecule (PECAM)	(Amabile *et al.*, 2008)
	CD62E*	E-selectin	(Amabile *et al.*, 2008)
	CD144	Vascular endothelial (VE)-Cadherin	(Faure *et al.*, 2006)
	CD106*	Vascular cell adhesion molecule 1 (VCAM-1)	(Tramontano *et al.*, 2010)
	CD146	Melanoma cell adhesion molecule (MCAM)	(Faure *et al.*, 2006)
	CD51	Integrin alpha V	(Sabatier *et al.*, 2002)
	CD105	Endoglin	(Bakouboula *et al.*, 2008)
Leucocyte-Derived MPs	CD45	Leucocyte common antigen	(Amabile *et al.*, 2008)
	CD62L*	L-selectin	(Priou *et al.*, 2010)
Granolocyte-Derived MPs	CD66B	Carcinoembryonic antigen-related cell adhesion molecule 8	(George, 2008)
Monocyte-Derived MPs	CD14	Lipopolysaccharide Receptor	(Shet *et al.*, 2003)
	CD142	Tissue Factor	(Aleman *et al.*, 2011)
T-cell Derived MPs	CD3	T-cell receptor	(Baka *et al.*, 2010)
	CD4		(Rautou *et al.*, 2012)
	CD8		(Kornek *et al.*, 2011)
RBC Derived MPs	CD235a	Glycophorin A	(Xiong *et al.*, 2012)

1.1.6. Functions of Microparticles

There are several reasons for cells to release MPs. They can act as cellular messengers by the transfer of membrane receptors, by the release of cytokines, and by the exchange of genetic information (Morel *et al.*, 2011b). MPs may also be 'cellular dustbins', allowing cells to remove dangerous components, such as caspase 3, which may otherwise accumulate (Abid Hussein *et al.*, 2007). The release of MPs may allow cells to escape phagocytosis by quickly removing 'eat me' signals, such as PS, from the cells surface. MPs are also implicated in several disease processes including inflammation, thrombosis, and endothelial dysfunction (Figure 1.2)

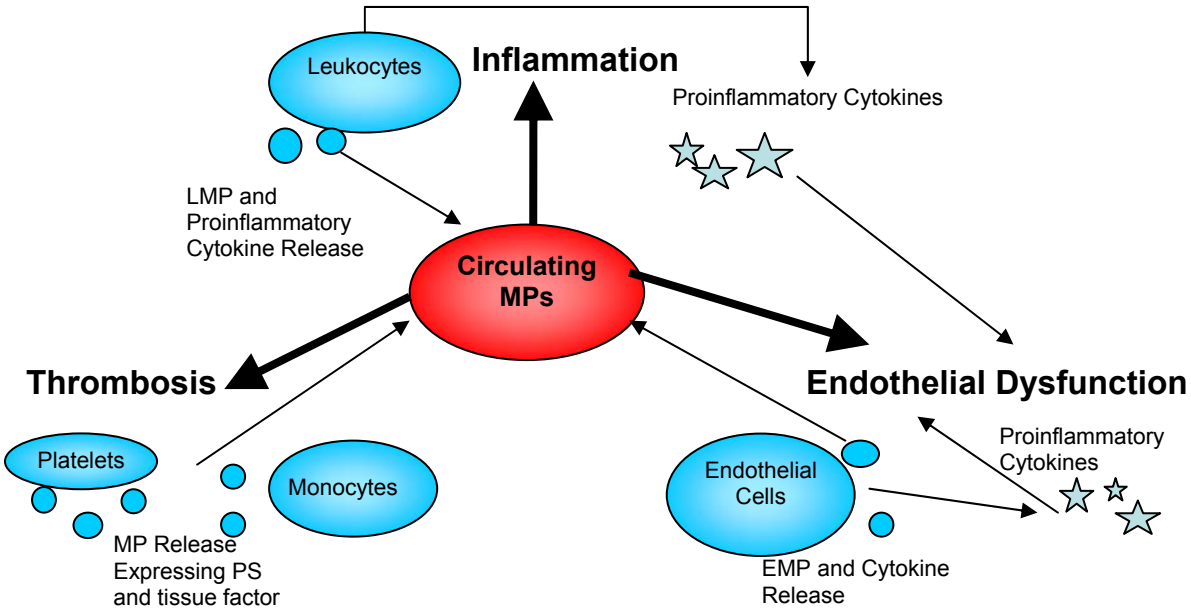

Figure 1.2 Functions of circulating microparticles from platelets, endothelial cells and leucocytes and interactions with proinflammatory cytokines

1.1.6.1. Inflammation

MPs are known to be elevated in many proinflammatory conditions and they have been shown to have potent proinflammatory activities. For example, PMPs promote leucocyte aggregation and binding of monocytes to endothelial cells (Nomura *et al.*, 2001), an early step in vascular inflammation.

PMPs trigger endothelial cells to release chemokines, which attract leucocytes to the endothelium (Nomura *et al.*, 2001). LMPs can stimulate proinflammatory gene expression in endothelial cells *in vitro,* leading to cytokine release and expression of adhesion molecules (Mesri and Altieri, 1999). Monocyte MPs can actually release IL-1β, which is derived from the original cell, giving an alternative mechanism for secretion of cytokines (MacKenzie *et al.*, 2001).

PMPs from rheumatoid arthritis patients have complement activators and activated complement components on their surface (Biro *et al.*, 2007), suggesting a role in the transport of inflammatory mediators.

MPs are also able to suppress the immune system, making their role in inflammation complex. Granulocyte-derived MPs contain functionally active Annexin 1, which gives them anti-inflammatory properties, such as down-regulating granulocyte activation, increasing IL-10 production, and inhibiting the interaction between leucocytes and endothelial cells *in vitro* (Dalli *et al.*, 2008).

1.1.6.2. Coagulation and Thrombosis

MPs provide a phospholipid surface for the assembly of blood coagulation factors, such as prothrombinase complexes, allowing for thrombin generation (Morel *et al.*, 2011a). Removing PMPs from plasma by high-speed centrifugation, prevents clotting of the plasma (Nieuwland and Sturk, 2007). The role of PMPs in coagulation is further demonstrated by the rare condition, Scott Syndrome, in which patients have bleeding complications due to impaired release of PMPs (Satta *et al.*, 1997). In autoimmune thrombocytopenia, elevated PMPs play a role in preventing bleeding complications (Jy *et al.*, 1992). It has been suggested that the membranes of PMPs have 50-100 fold higher procoagulant activity than that of activated platelets (Sinauridze *et al.*, 2007).

The role of MPs in coagulation is however complex. MPs can express PS and tissue factor, central components of coagulation. Conversely, PMPs can inactivate coagulation factors Va and VIIIa, suggesting that they also possess anti-coagulation properties (Tans *et al.*, 1991). In healthy individuals, the level of annexin V+ MPs is inversely correlated to the plasma concentration of thrombin-antithrombin complex, suggesting that in this case MPs have an anticoagulant function (Berckmans *et al.*, 2001). Whereas under pathological conditions, the number of MPs exposing tissue factor can be elevated (Bakouboula *et al.*, 2008), leading to increased coagulation.

The procoagulant potential of MPs is not restricted to PMPs. EMPs express von Willebrand factor, resulting in platelet aggregate formation (Jimenez *et al.*, 2003a) and LMPs can transfer tissue factor between platelets and trigger thrombosis (Meziani *et al.*, 2008). In fact, at the onset of coagulation, monocyte-derived MPs are the main contributor of tissue factor activity, and are crucial for thrombus formation, whereas PMPs may be more of an indicator of platelet activation (Morel *et al.*, 2011b). RBC-derived MPs may also be important contributors to coagulation in sickle cell disease (van Beers *et al.*, 2009).

1.1.6.3. Endothelial Dysfunction

Normal endothelial function involves control of vasodilation and the immune function of the endothelium. Endothelial dysfunction refers to a loss of normal homeostatic

functions in the blood vessels and is characterized by a shift of the endothelium toward vasoconstriction, a proinflammatory state, and chronic prothrombic properties. These are key features in early atherosclerosis development and predate clinical symptoms by many years (Bonetti *et al.*, 2003). Endothelial dysfunction promotes atherosclerosis by causing upregulation of adhesion molecules, increased cytokine secretion and platelet activation (Verma and Anderson, 2002).

EMPs both reflect endothelial dysfunction, and can themselves induce it. EMPs are released from the endothelium in response to various stimuli, such as proinflammatory cytokines (Diamant *et al.*, 2004). EMPs are in turn capable of impairing vasodilatation, by altering the release of nitric oxide (NO) and reactive oxygen species (Brodsky *et al.*, 2004). MPs from metabolic syndrome patients reduce NO production and impair endothelium-dependent relaxation when injected into mice (Agouni *et al.*, 2008).

Elevated MP levels have been observed in pathologies associated with endothelial dysfunction, including acute coronary syndromes and hypertension (VanWijk *et al.*, 2003). Correlations have been found between EMP levels and other markers of endothelial dysfunction, such as reduced flow-mediated brachial artery dilation and increased arterial stiffness (Amabile *et al.*, 2005).

Again the relationship between MPs and endothelial dysfunction is made some what unclear by the fact that, in some cases, MPs may improve endothelial dysfunction. For example, elevated circulating MPs have been found to be protective against vascular hyporeactivity in sepsis (Mostefai *et al.*, 2008b).

1.1.6.4. Summary of Microparticle Functions

The role of MPs in inflammation, coagulation and endothelial dysfunction is still not fully understood. Contradicting reports suggest that MPs have both a beneficial and a harmful role to play in these processes. It may be that a careful balance of MPs of different subtypes is required for homeostasis, and when this balance is disturbed by disease, MPs switch to having a pathological role.

1.1.7. Clearance of Microparticles

The presence of MPs in plasma reflects a balance between MP production by activated and apoptotic cells, and their clearance from the circulation. It was thought that, due to their smaller size and greater ability to diffuse and escape phagocytosis, MPs may be able to survive in the circulation longer than the cells they originate from (Freyssinet, 2003). However there is limited information on the mechanisms of clearance.

MPs are thought to be cleared from the circulation by phagocytes in the liver or spleen. Phagocytes can recognise PS expressing MPs by receptors that bind PS directly or by receptors for the proteins that opsonize MPs (Flaumenhaft, 2006).

It has been shown that procoagulant PMPs are rapidly removed from the circulation following injection into rabbits (Rand *et al.*, 2006), and within 30 min following infusion in mice (Flaumenhaft, 2006). Rank *et al* demonstrated that the half life of annexin V+ MPs in humans is 5.8 hours following transfusion of platelet concentrates, and that the removal of PMPs was quicker than the removal of platelets (Rank *et al.*, 2011a).

Defects in the clearance of MPs can lead to problems, including increased risk of thrombosis. Splenectomised mice have a defect in clearing infused tumour cell MPs, and therefore have increased mortality compared to non-spenectomised mice (Davila *et al.*, 2008).

1.2. Other Inflammatory Markers

1.2.1. Cytokines

Cytokines are small, hormone-like, signalling molecules that modulate the behaviour of cells within the immune and haematopoietic systems. They act by binding specific receptors, inducing signal transduction pathways, leading to activation of effector mechanisms in target cells (Ait-Oufella *et al.*, 2011). Cytokines share many characteristics with hormones and growth factors, other soluble factors that have biological effects (Goldsby *et al.*, 2003). However, growth factors are produced constitutively, whereas cytokines secretion is in response to a stimulus and is usually short-lived.

There are over 200 different cytokines (Girn *et al.*, 2007), which can by classified by the structural homology of their receptors; Class I family, Class II family, IL-1 family, Tumour Necrosis Factor (TNF) family and TGF family (Tedgui and Mallat, 2006). Cytokines can also be classified into functional categories. They are currently grouped by those which enhance cellular immune responses (Th1), antibody responses (Th2) or inflammation (Th17) (Stummvoll *et al.*, 2008). Chemokines are a group of cytokines that mediate movement of cells around the body and direct leucocytes to sites of inflammation (Girn *et al.*, 2007).

Once released, cytokines can act on the same cell (autocrine), neighbouring cells (paracrine) or rarely systemically (endocrine) (Tedgui and Mallat, 2006). Cytokine signalling can lead to cell survival, proliferation, release of the same or other cytokines, cell differentiation or cell death.

One cytokine can have different biological effects on different target cells. There is often redundancy in cytokines, as two or more cytokines may mediate similar functions. Cytokines can exhibit synergy, as the combined effect of two cytokines on a cell is greater than the effect of individual cytokines (Tedgui and Mallat, 2006). Conversely, one cytokine can antagonise another's effect (Bhandari and Elias, 2006).

Cytokines act by binding to cytokine receptors expressed on cell surfaces. The α-chain of a cytokine receptor is associated with a family of tyrosine kinases, the Janus kinase (JAK) family (Usacheva *et al.*, 2002). Cytokine binding induces the association of receptor subunits and the activation of JAKs. Activated JAKs create sites for signal transducers and activators of transcription (STAT) transcription factors by phosphorylation of specific tyrosine residues on cytokine receptor subunits. STAT transcription factors then translocate from the docking sites at the membrane to the nucleus, where they initiate the transcription of specific genes (Goldsby *et al.*, 2003).

1.2.2. C-Reactive Protein (CRP)

CRP is an acute phase protein, synthesised by hepatocytes. The acute phase response occurs during acute and chronic inflammation, including infection, tissue injury, autoimmunity and malignancy. IL-6 (produced predominantly by macrophages and adipocytes) and other cytokines, trigger the release of CRP (Pepys and Hirschfield, 2003). IL-6 levels have been shown to be correlated with CRP levels (Kasperska-Zajac *et al.*, 2011). CRP rapidly increases following infection or cell damage, and it reaches a peak at 48hrs. CRP levels can increase 10,000 fold in response to infection (Pepys and Hirschfield, 2003).

CRP is recognised by C1q, activating the classical complement pathway and enhancing phagocytosis (Pepys and Hirschfield, 2003). Therefore, it is an important component in the effective clearance of dying cells, by acting as an opsonin.

1.2.3. Microparticles and Other Inflammatory Markers

The relationship between MPs and cytokines can be both causal and consequential. Thus MPs can be formed following cell activation by inflammatory cytokines, such as IL-1β and IL-6, and MPs can also stimulate the release of cytokines. *In vitro* stimulation with PMPs results in production of IL-8, IL-1β and IL-6 from endothelial cells (Nomura *et al.*, 2001).

MPs are able to both stimulate and suppress immune responses, at different stages of inflammation. In the early stages, neutrophil-derived MPs are able to induce release of anti-inflammatory cytokines, including TGF-β (Gasser and Schifferli, 2004). However,

later in inflammation, MPs cause release of proinflammatory cytokines, including IL-6 from endothelial cells (Mesri and Altieri, 1999).

MPs with bound CRP have been identified in the plasma of patients with rheumatoid arthritis (Biro *et al.*, 2007) and acute myocardial infarction (van der Zee *et al.*, 2010). CRP-bound MPs were found to be correlated with MPs that bound C1q, the first component of the classical pathway of complement activation, in patients undergoing cardiac bypass surgery (Biro *et al.*, 2011).

In a study assessing the subclinical atherosclerosis burden in asymptomatic individuals, high levels of CRP were associated with increased levels of LMPs (Chironi *et al.*, 2006). CRP has been noted to induce the release of EMPs both *in vitro* and *in vivo* (Devaraj *et al.*, 2011).

The relationship between MPs and other inflammatory markers is complex and may be further complicated in various disease states. There is growing interest in the investigation of inflammatory biomarkers and their interactions with each other, particularly in cardiovascular disease (CVD).

1.3. Cardiovascular Disease

CVD is any disease of the cardiovascular system, including coronary artery disease (CAD), hypertension, cerebrovascular disease, congenital heart disease, shock and heart failure. There are also several diseases that are known to be associated with CVD, including diabetes, obstructive sleep apnoea and preeclampsia, which often occur prior to CVD.

Although CVD refers to any disease that affects the heart or vascular system, it is often used to describe the pathologies caused by atherosclerosis. Atherosclerosis is a progressive hardening and narrowing of the arteries, caused by the formation of plaques on the inside surface of the arteries.

1.3.1. Inflammation and the Development of Atherosclerosis

In healthy individuals, endothelial cells usually resist leucocyte adhesion. Certain conditions such as a high-fat diet, obesity and smoking can trigger the endothelium to express adherence molecules, such as P-selectin and VCAM-1 (Demerath *et al.*, 2001).In the early stages of atherosclerosis, selectins mediate loose rolling of leucocytes, and integrins mediate firm attachment. Endothelial cells secrete chemokines, which direct migration of attached cells into the intima (Libby *et al.*, 2002).

As atherosclerosis progresses monocytes mature into macrophages, which capture oxidised low-density lipoprotein (LDL), converting them into foam cells. These cells proliferate and secrete TNF-α and IL-1β. T cells then enter the intima and secrete Th1 cytokines, including IFN-γ (Robertson and Hansson, 2006). Smooth muscle cells (SMCs) express enzymes that degrade elastin and collagen, allowing more cells to enter the expanding plaque (Packard and Libby, 2008).

Inflammatory mediators, such as IFN-γ, inhibit collagen synthesis, making the fibrous cap weak. When the cap ruptures, tissue factor triggers the thrombus formation, which causes the arterial occlusion and the acute complications of atherosclerosis. Therefore, inflammation is pivotal in all stages of atherosclerosis, from lesion initiation to destabilization (Packard and Libby, 2008) (Figure 1.3).

Atherosclerosis can develop silently years in advance of any coronary event, but there are currently limited mechanisms to detect this early development. Imaging techniques can miss the early stages and would be too expensive and time consuming to perform on a large population. Therefore, the identification of circulating biomarkers which indicate atherosclerosis is of much interest.

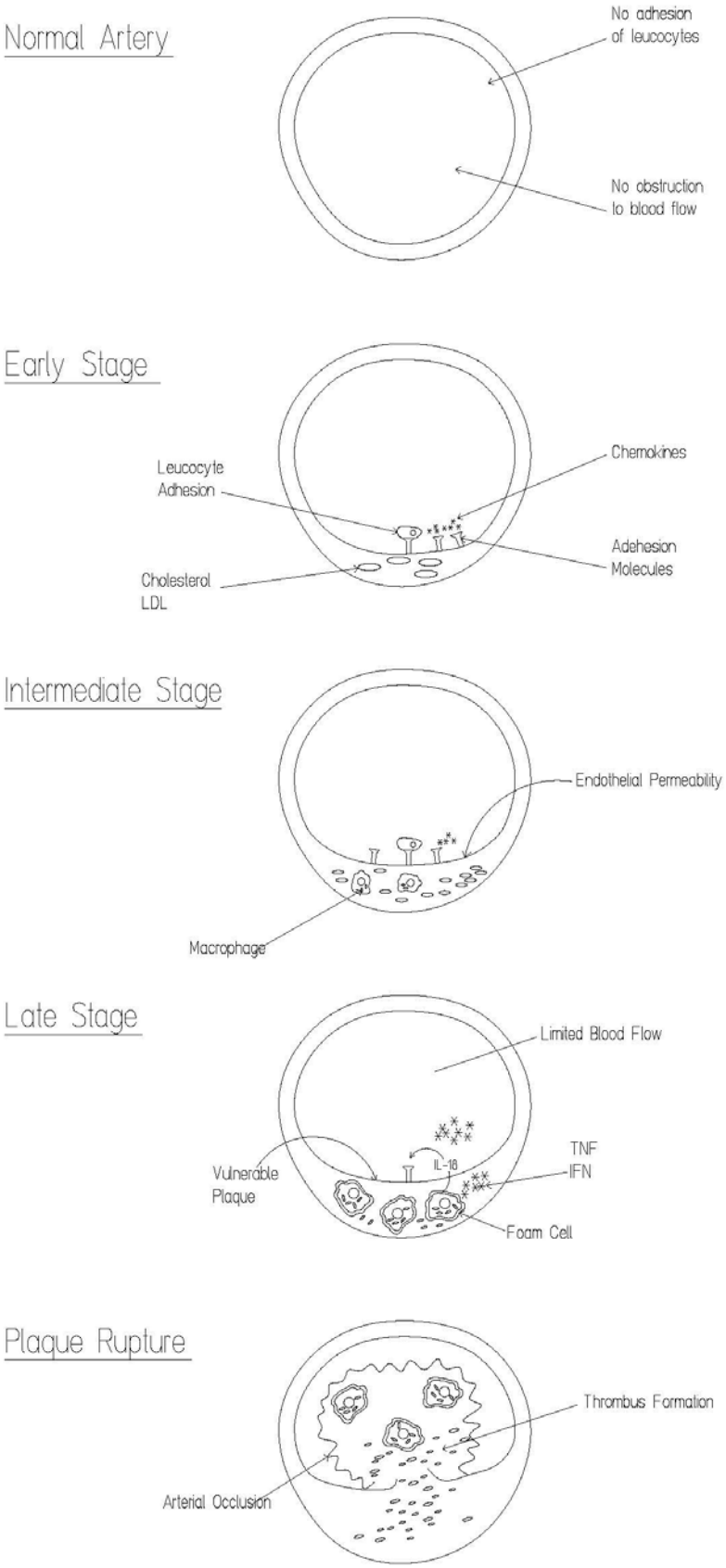

Figure 1.3 Stages in the development of atherosclerosis, from the early stage of leucocyte adhesion to plaque rupture and thrombus formation

1.3.2. Microparticles in Cardiovascular Disease

MPs may provide a potential link between the processes of inflammation, thrombosis and endothelial dysfunction, and the development of atherosclerosis, because they express both proinflammatory and pro-thrombotic molecules on their surface and they can influence endothelial function.

Many studies have found elevated MP levels in CVD, including acute coronary syndromes (Bernal-Mizrachi *et al.*, 2004; Mallat *et al.*, 2000) and venous embolism (Chirinos *et al.*, 2005a), as well as in diseases associated with CVD, such as type 2 diabetes (Feng *et al.*, 2009) and preeclampsia (Lok *et al.*, 2008).

It is now understood that CVD is primarily a disease of inflammation. MPs are mediators of inflammation, for example, PMPs can trigger release of IL-6 from endothelial cells, inducing the expression of adhesion molecules on endothelial cells *in vitro* (Nomura *et al.*, 2001). PMPs also provide IL-1β, a proinflammatory cytokine, which contributes to endothelial inflammation (Lindemann *et al.*, 2001).

Patients with CVD have an increased risk of arterial thrombosis, which is demonstrated in acute myocardial infarction and stroke. MPs are a crucial component in the coagulation cascade and elevated levels in CVD may provide a mechanism for the increased risk of thrombosis. Apoptotic MPs are present in high concentrations within human atherosclerotic plaques and they are responsible for the majority of tissue factor activity, suggesting that these MPs may largely determine plaque thrombogenicity (Mallat *et al.*, 2000).

Endothelial dysfunction is a feature of atherosclerosis (Neunteufl *et al.*, 1997), hypertension (Contreras *et al.*, 2000) and preeclampsia (Roberts, 1998). Certain subtypes of MPs have been shown to cause endothelial dysfunction. Circulating apoptotic MPs have been shown to correlate with the impairment of coronary endothelial function in patients with CAD. Annexin V+CD31+ MPs are able to predict severe endothelial dysfunction, even when controlling for other risk factors (Werner *et al.*, 2006). MPs may play an important role in the initiation and development of atherosclerosis, as well as accelerating the progression of the atherosclerotic plaque.

PMPs have been shown to induce angiogenesis, both *in vitro* and *in vivo* (Brill *et al.*, 2005). Although angiogenesis can be beneficial by providing alternative pathways for blood supply within an ischemic myocardium, it can also increase plaque instability (Virmani *et al.*, 2005). MPs from atherosclerotic plaques, expressing CD40L on their surface, have been shown to stimulate endothelial cell proliferation, potentially increasing plaque vulnerability (Leroyer *et al.*, 2008a).

1.3.3. Other Inflammatory Markers in Cardiovascular Disease

Cytokines regulate every stage of the development of atherosclerosis, from endothelial activation, to the adhesion of inflammatory cells and the subsequent remodelling of the vascular environment. IL-6 enhances the expression of cell adhesion molecules on endothelial cells and SMCs, as well as increasing production of acute phase reactants, such as CRP (Haddy *et al.*, 2003). TNF-α induces both a proinflammatory and pro-thrombotic local environment in the vessel (Leon and Zuckerman, 2005). TNF-α, IFN-γ and IL-1 levels are all associated with an increased tendency for plaque rupture. IFN-γ potentially destabilises the fibrous cap by inhibiting SMC proliferation (Girn *et al.*, 2007).

Conversely, some cytokines offer protection from the development of atherosclerosis. IL-10 can reduce atherosclerosis, by inhibiting the release of proinflammatory cytokines and by suppressing CD40/CD40L mediated responses (Terkeltaub, 1999). TGF-β inhibits SMC proliferation, as well as reducing proinflammatory cytokine release. In fact, there is a negative correlation between TGF-β levels and CAD (Erren *et al.*, 1999).

Increased CRP is predictive of future atherothrombotic events, including myocardial infarction and stroke (Koenig *et al.*, 1999; Ridker, 2003). CRP is deposited in the area surrounding a plaque and contributes to myocardial damage, by promoting complement activation (Nijmeijer *et al.*, 2004). The measurement of CRP is more convenient than other inflammatory markers, due to its stability in serum and the availability of robust and standardised assays. High-sensitivity CRP assays are also available, allowing the detection of slightly elevated levels, which is useful in identifying individuals at low to moderate risk of CVD (Ridker and Cook, 2004).

CRP levels are strongly positively associated with BMI, which may be partly due to the release of IL-6 from adipocytes (Yudkin *et al.*, 1999). Elevated CRP is also linked with the risk of metabolic syndrome and diabetes mellitus, both cardiovascular-associated conditions (Pepys and Hirschfield, 2003). Statins reduce circulating CRP levels (Ridker *et al.*, 2001), explaining one of their mechanisms of action to reduce CVD risk.

Levels of CRP and inflammatory cytokines have been proposed as useful predictors of future CVD risk (Harris *et al.*, 1999; Ridker, 2003). Cytokine and CRP levels may also be beneficial in monitoring the response to treatments of atherosclerosis (Girn *et al.*, 2007).

1.4. Aims of the Project

1) To adapt and fully validate a flow cytometric assay to measure all MP subtypes described above, and to address the issue of pre-analytical and analytical sources of variability in the measurement of MPs.

2) To validate the cell specific markers used in this study by generating MPs *in vitro* using a variety of agonists, and to determine the impact of agonist used on the phenotype of EMP released.

3) To investigate alternative techniques for MP analysis and examine associations between results from quantitative and functional assays.

4) To measure levels of MPs in healthy individuals to establish a normal range, to analyse variability in levels based on age and sex, and to determine the impact of hypoxia on levels of MPs in healthy individuals.

5) To determine the impact of therapy on levels of MPs and other inflammatory markers in obstructive sleep apnoea patients.

6) To examine levels of circulating MPs in cardiovascular-associated diseases, to investigate MP levels 5-10 years following a preeclamptic pregnancy, and to determine if circulating MP levels provide additional value in the diagnosis of atherosclerosis by dobutamine stress echocardiogram.

Chapter 2 – Validation of a Flow Cytometric Assay to Measure Circulating Cell-Derived Microparticles

2.1. Introduction

Despite the growing interest in MPs in the pathogenesis of disease, there is a lack of standardisation when it comes to their measurement (Hind *et al.*, 2010). The variety of methods used by different laboratories is a major barrier to the detection and understanding of MPs role in disease (Shet, 2008). Details on the optimal technique for preparing, processing and storing blood samples, along with the influence of different analytical techniques on MP counts are sparse (Shah *et al.*, 2008). In the past few years, efforts have been made to standardise both the pre-analytical and analytical steps. However, published studies still use a wide range of methods, and consequently report widely varying levels of MPs, even in similar patient groups.

2.1.1. Plasma Samples for Measurement of Microparticles

Circulating cell-derived MPs are detected in plasma, which is obtained by centrifugation of blood samples. Citrate is the preferred anticoagulant for MP analysis, because of its ability to chelate calcium, which is necessary for MP release (Hind *et al.*, 2010). Ideally all platelets would be removed from samples prior to freezing or analysis, by a series of centrifugations, to prevent *ex vivo* PMP production. However, this is likely to result in the loss of some larger MPs, as platelets and MPs may form a continuum in size (Lynch and Ludlam, 2007). In practice, a variety of centrifugation protocols are used by different groups, which produce platelet-poor-plasma (PPP), as opposed to platelet-free-plasma.

MPs can be isolated from PPP by high-speed centrifugation, before re-suspension in a filtered buffer (Lynch and Ludlam, 2007). This allows MPs to be washed prior to analysis, to remove lipids, such as chylomicrons, which are a similar size to MPs and cause significant background noise when analysing MPs by flow cytometry. However, there is no consensus on the optimal centrifugation speed to pellet all MPs.

2.1.2. Flow Cytometry Measurement of Microparticles

Flow cytometry has been the most commonly used method for the detection of MPs, with approximately 75 % of laboratories using it for clinical samples (Lacroix *et al.*, 2010). PPP is incubated with fluorescence-conjugated antibodies to cell-specific markers. MPs are identified on a Forward Scatter (FSc) vs. Side Scatter (SSc) plot, using size-calibrated beads to define the MP gate. Flow cytometry can identify MPs from different cellular origins, with the use of cell-specific markers. It can also be quantitative, if the flow rate for the machine is determined or if counting beads are used.

Calibrated beads with defined diameters can be used for the adjustment of instrument settings, and to define and standardise MP size gates. However, the refractive index of plastic beads differs from that of MPs, making them an imperfect method for defining size cut-offs (Yuana *et al.*, 2011).

There are many different flow cytometry protocols used for the analysis of MPs, which vary greatly in their methods for blood collection, centrifugation, storage of samples and the cell-specific antibody markers used (Jy *et al*, 2004). This has led to a great variation in the reported levels of MPs in the literature. Standardisation of MP measurement will allow for the comparison of results between research groups, and for a greater understanding of the role of MPs in health and disease.

2.1.3. Aims of this Chapter

1) To adapt a flow cytometric assay for the measurement of a wide range of MP subtypes.

2) To validate this assay by determining the linearity, intra-assay precision and inter-assay precision.

3) To identify potential causes of pre-analytical and analytical variability in MP quantification using this assay, and to use these findings to make recommendations towards the standardisation of MP analysis.

2.2. Methods

2.2.1. Subjects

28 healthy controls were used for the validation of procoagulant MPs, PMPs and RBC-derived MPs. They were recruited from laboratory staff. Subjects were eligible if aged 18 or above and in good health. Subjects with a known cardiovascular condition or a known cardiovascular-associated condition were excluded. This study was approved by the Oxford Research Ethics Committee (REC No: 11/SC/0183).

56 obstructive sleep apnoea (OSA) patients were used for validating EMP, LMP, granulocyte, monocyte and T-cell-derived MP measurements, as they are only found at very low levels in healthy individuals (VanWijk *et al.*, 2002). Patients were eligible if they were aged between 45 and 75 years, had proven OSA. This study was approved by the Oxford Research Ethics Committee (REC No: 05/Q1604/159).

Written informed consent was obtained from all participants.

2.2.2. Flow Cytometric Method for Detection of Microparticles

2.2.2.1. Preparation of Microparticles

A flow cytometric assay was adapted from the method originally described by Biro *et al* (2004). Blood from healthy controls and OSA patients was drawn into citrate vacutainer tubes (Becton Dickinson (BD), Oxford, UK) using a 19 G needle (BD, Oxford, UK). The first 3 ml of blood following venepuncture was discarded, to avoid contamination from EMPs due to vascular damage. Within 15 minutes of the blood being taken, tubes were centrifuged at 1550 g for 20 min at 20 °C, to produce PPP. PPP was carefully removed from the top of the tube, without disturbing the buffy coat, and 250 µl aliquots frozen immediately and stored at -80 °C. The method described by Biro *et al* recommends snap freezing in liquid nitrogen, prior to storing at -80 ºC. However, a lack of facilities prevented this step.

When required the 250 µl aliquots were thawed on melting ice and then centrifuged at 18,000 g for 30 min at 20 °C. 225 µl of supernatant was removed, and the pellet was re-suspended in 225 µl of phosphate-buffered saline (PBS)-citrate 0.32 %. The samples were centrifuged again at 18,000 g for 30 min at 20 °C. 225 µl of supernatant was removed and the pellet was re-suspended in 75 µl of PBS-citrate 0.32 %, making the final MP suspension 2.5 times concentrated from the original PPP.

2.2.2.2. MegaMix Beads

To establish if the BD FACsCalibur flow cytometer (BD, Oxford, UK) used in this study was capable of resolving particles of 0.5 μm and 0.9 μm in size, MegaMix beads (BioCytex, Marseille, France) were used. They are a mixture of fluorescent beads with defined diameters; 0.5 μm, 0.9 μm and 3.0 μm, that allow for standardisation of instrument settings on different flow cytometers. MegaMix Beads were acquired by flow cytometry according to the manufacturer instructions.

2.2.2.3. Establishing a Microparticle Gate

Both FSc and SSc were set to a logarithmic gain. To establish a suitable MP gate on a flow cytometry plot of FSc vs. SSc, it was necessary to distinguish between MPs and small platelets. Platelet-rich-plasma (PRP) was therefore prepared by centrifugation of citrate blood at 200 g for 10 min. The PRP was analysed on a BD FACSCalibur to establish a platelet gate. PRP was then incubated with 0.35 μg/ml calcium ionophore A23187 (Sigma, Kent, UK) for 5 hours. Calcium ionophore is known to stimulate platelets to release MPs (Nieuwland and Sturk, 2007; VanWijk *et al.*, 2003; Yin *et al.*, 2008). The stimulated PRP was analysed to establish a MP gate. The threshold was set on SSc so to exclude background noise, which was determined by running 0.22 μm filtered PBS-calcium (2.5 nmol/L) with no added sample. A fluorescence threshold was avoided to allow analysis of both the annexin V positive and negative MPs. This MP gate was used for all future flow cytometric experiments.

2.2.2.4. Selection of Surface Markers for Microparticle Subtypes

AnnexinV-fluoresceinisothiocyanate (FITC) and lactadherin-FITC both bind to PS, so were used to stain procoagulant MPs. PECAM (CD31)-phycoerythrin (PE) and platelet glycoprotein II (CD41)-phycoerythrin-Cy5 (PE-Cy5) were used to differentiate between PMPs (CD31+CD41+) and EMPs (CD31+CD41-). VE-Cadherin (CD144)-PE, E-selectin (CD62E)-PE-Cy5 and VCAM-1 (CD106)-PE-Cy5 were all used as a markers for EMPs. Leucocyte common antigen (CD45)-allophycocyanin (APC) was used as a marker for total LMPs. Carcinoembryonic antigen-related cell adhesion molecule 8 (CD66B)-FITC was used to stain granulocyte-derived MPs. Lipopolysacharride receptor (CD14)-PE was used to identify monocyte-derived MPs. T-cell receptor (CD3)-Peridinin Chlorophyll Protein (PerCP) was used to label T-cell-derived MPs. Glycophorin-A (CD235a)-PE was used to stain RBC-derived MPs. All antibodies were supplied by BD (Oxford, UK), except for lactadherin (HTI, Vermount, USA) and CD144 (eBioscience, Hatfield, UK).

Antibodies were diluted in PBS-calcium 2.5 nmol/L (PBS-Ca) to an optimal concentration established by titration of each batch, with either samples of a known high MP count, or on the supernatant from stimulated cells. The following final concentrations

were used: annexin V (0.10 μg/ml), lactadherin (1.38 μg/ml), CD31 (0.10 μg/ml), CD41 (0.013 μg/ml), CD144 (4.17 μg/ml), CD62E (0.42 μg/ml), CD106 (0.21 μg/ml), CD45 (0.21 μg/ml), CD66B (0.10 μg/ml), CD14 (0.42 μg/ml), CD3 (0.10 μg/ml), glycophorin A (0.069 μg/ml), IgG_1-PE (0.42 μg/ml), IgG_1-PECy5 (0.052 μg/ml) and IgG_1-APC (0.83 μg/ml). Diluted monoclonal antibodies were centrifuged at 18,000 g for 5 min to remove protein aggregates.

2.2.2.5. Labelling of Microparticles

Annexin V-FITC in the absence of calcium and appropriate isotype control antibodies were used as negative controls. 5 μl of diluted annexin V-FITC, lactadherin-FITC or antibodies were added to PBS-Ca (2.5 mmol/L) to make up a total volume of 50 μl. 10 μl of concentrated PPP sample was added and incubated with the appropriate antibodies for 30 min at room temperature, protected from light. After the incubation, 900 μl of PBS-Ca was added.

2.2.2.6. Acquisition and Analysis of Microparticles on the BD FACSCalibur

The MP gate was checked at the beginning of each assay by measuring a tube containing 10 μl of PPP and 5 μl 1:1000 dilution of 1 μm beads (Sigma L-2778) in 950 μl of PBS-calcium (2.5 mmol/L). The flow rate of the FACSCalibur was determined daily by acquiring a weighed tube of PBS-Ca for 10 min, then weighing the tube and calculating the volume of liquid taken up. This figure is divided by 10 to give the flow rate μl per min.

MP numbers per μl were calculated using the following formula:

$$\frac{\text{Event Count} \times \%\ \text{Subgroup}}{\text{Flow Rate (μl per min)} \times \text{Time of Acquisition (2 min)}} \times \frac{\text{Total Volume in Tube (960 μl)}}{\text{Vol of MP Added (10 μl)}} \times \frac{\text{Vol of Conc Sample (100 μl)}}{\text{Vol of Original Aliquot (250 μl)}} = \text{MP/μl}$$

2.2.2.7. Single Colour Staining and Fluorescence-Minus-One (FMO) Staining

Compensation settings were established by acquiring single-colour stained tubes. Each individual fluorochrome-conjugated antibody was used to stain MPs separate from other antibodies. Any fluorescent cross-talk into inappropriate channels was adjusted using the compensation settings. These settings were saved for future experiments, and settings were checked every month.

Isotype control antibodies were used for each fluorochrome to assist in the gating of negative and positive events. However, as it has been previously noted that the diversity in staining intensity of isotype controls can lead to a large errors in the quantification of MPs (Trummer *et al.*, 2008), an alternative method for establishing gates was also used. FMO staining involves adding all fluorochrome-conjugated antibodies to a tube minus the antibody of interest. This allows for a negative/positive gate to be drawn based on the background staining from the other fluorochromes in the tube (Baumgarth and Roederer,

2000). FMO staining was carried out every month to check gates established by isotype controls.

2.2.2.8. Flow Rate calculated by Weighed Tube Method versus TruCount Tube Method

In order to give an absolute MP count it is necessary to calculate the flow rate of the flow cytometer. This can vary over time, so is measured for each run of MP samples. The flow rate can be calculated by weighed tube, as described above, or it can also be calculated by acquiring calibrated counting beads in a known volume of PBS-Ca for 10 min, then counting the beads acquired to calculate the µl per min. The weighed tube method was compared to the calibrated counting beads method (TruCount tubes, BD, Oxford, UK) on 10 separate days.

2.2.3. Validation of the Assay

PPP from healthy controls was used to validate procoagulant MPs, PMPs and RBC-MPs. However, to assess the linearity, intra-assay and inter-assay precision of EMPs, LMPs, granulocyte-derived MPs and monocyte-derived MPs it was necessary to use alternative sources, as healthy controls have very low numbers of these MPs. For this reason, PPP from OSA patients, with higher levels of these MPs, were used for EMP, LMP, granulocyte-derived MP and monocyte-derived MP populations.

2.2.3.1. Linearity

40 ul, 20 µl, 10 µl, 5 µl, 2.5 µl 1.25 µl and 0.625 µl of sample was labelled with the appropriate antibodies to identify different populations of MPs and then analysed by flow cytometry, using the protocol described above.

2.2.3.2. Intra-Assay Precision

Intra-assay precision was established by analysing a single sample 18 times on the same day. MPs were analysed using the protocol described above.

2.2.3.3. Inter-Assay Precision

Several pooled aliquots of PPP from healthy controls and from OSA patients were prepared and frozen at -80 °C. Each aliquot was thawed and used only once. Inter-assay precision was established by running these samples on each MP assay. This was carried out 12 times. MPs were analysed using the protocol described above.

2.2.4. Sources of Variability within the Assay

For the sources of variability experiments, only annexin V+ MPs, CD31+CD41+ PMPs, CD31+CD41- EMPs, CD144+ EMPs, CD62E+ EMPs, CD106+ EMPs and CD45+ LMPs were analysed.

2.2.4.1. Effect of Time before Centrifugation

Eight blood samples were taken, from eight healthy controls. Citrate blood samples were left standing upright in a rack undisturbed for the following amount of time, before centrifugation: 15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 105 min and 120 min. The number of annexin V+ MPs and PMPs were then analysed 8 times, according to the above protocol. The first tube following venepuncture was discarded, and subsequent tubes were randomised, so that any differences found would not be due to venepuncture order.

2.2.4.2. Effect of Washing Platelet-Poor-Plasma Samples

Ten healthy controls had two 250 µl aliquots of PPP stored at -80 ºC for 1 month and thawed on melting ice. One aliquot for each control was washed by centrifugation at 18,000 g for 30 min, after which 225 µl of supernatant was removed and 225 µl of PBS-citrate (0.32%) added. Then centrifuged again at 18,000 g for 30 min, 225 µl of supernatant was removed and 75 µl of PBS-citrate (0.32%) was added. The second aliquot was labelled for MPs without any washing steps.

2.2.4.3. Effect of Single versus Double Centrifugation

Two citrate blood samples were taken from 18 healthy controls. One tube was centrifuged at 1550 g for 20 min, as per a single centrifugation protocol. The other tube was centrifuged at 1550 g for 20 min, followed by a second centrifugation at 13,000 g for 2 min. Aliquots of PPP were taken from each tube and were immediately frozen at -80 ºC. Samples were then processed according to the above protocol.

2.2.4.4. Fresh versus Frozen Samples

Citrate blood was obtained from 10 healthy controls, and was spun at 1550 g for 20 min, followed by a second centrifugation at 13,000 g for 2 min. Two 250 µl aliquots of PPP were taken for each control. One was stained and analysed within 3 hours and the second was placed in a -80 °C freezer, stored for one month then thawed on ice and analysed.

2.2.4.5. Effect of Thawing Temperature on MP Samples

Citrate blood was taken from 10 healthy controls, and centrifuged at 1550 g for 20 min, followed by a second centrifugation at 13,000 g for 2 min. Three aliquots of PPP were taken and frozen at -80 ºC. The samples were thawed on melting ice, at room temperature (RT) or at 37 ºC, then washed, labelled and analysed.

2.2.4.6. Effect of Long-term Storage at -80 ºC of MP Samples

56 OSA patients had 2 aliquots of single centrifuged PPP stored at -80 ºC for MP analysis. The first aliquots were thawed and analysed within 12 months of storage. The second aliquots were stored at -80 ºC for an additional 20 months before they were thawed and analysed, according to the above protocol.

2.2.4.7. Summary of Sources of Variability Experiments

Table 2.1 – Summary of Sources of Variability Experiments

Experiment	Samples Used	Difference in Method
Effect of Time before Centrifugation	8 Healthy Controls	Blood samples left 15, 30, 45, 60, 75, 90, 105, 120 minutes between venepuncture and centrifugation
Effect of Washing PPP Samples	10 Healthy Controls	PPP washed twice at 18,000 g for 30 min to pellet MPs versus no washing step
Effect of Single versus Double Centrifugation	18 Healthy Controls	Blood samples centrifuged at 1550 g 20 min or at 1550 g 20 min plus 13,000 g 2 min
Fresh versus Frozen Samples	10 Healthy Controls	PPP samples analysed either fresh or after one month stored at -80 ºC
Effect of Thawing Temperature	10 Healthy Controls	PPP samples were thawed either on melting ice, at RT or at 37 ºC
Effect of Long-term Storage at -80 ºC	56 OSA Patients	PPP samples analysed within 12 months storage, or after an additional 20 months storage

2.2.5. Statistics

All statistics were performed using GraphPad Prism 5 Software (GraphPad Software, San Diego). The linearity of the MPs subtypes was assessed by linear regression. The percentage coefficient of variance (CV%) value for intra-assay and inter-assay precision was calculated for each of the MP subtypes. The effect of time before centrifugation was assessed by a repeated measures ANOVA, followed by the post hoc test for a linear trend. The effect of thawing temperature was assessed by repeated measures ANOVA. Paired results in the effect of washing, single versus double centrifugation, fresh versus frozen, and TruCount versus weighed tube experiments were compared by Wilcoxon Signed Rank test. A p-value of <0.05 was considered statistically significant.

2.3. Results

2.3.1. Flow Cytometric Method for Detection of Microparticles

2.3.1.1. MegaMix Beads

Conventional flow cytometers are designed to measure cells, and often they are unable to detect smaller particles. So it was necessary to establish if the flow cytometer (BD FACSCalibur) used in this study would be capable of resolving small particles. MPs range from 0.1 µm to 1.0 µm in size, although conventional flow cytometers are not usually considered to be able to detect particles much smaller than 0.5 µm .

MegaMix beads were used to determine if the BD FACSCalibur was capable of resolving beads between 0.5 µm and 0.9 µm in size. Figure 2.1A shows that the three size calibrated beads, 0.5 µm, 0.9 µm and 3.0 µm can be discriminated on a SSc vs FL1 plot. Figure 2.1B shows that 0.5 µm and 0.9 µm beads are clearly discriminated on SSc and partially discriminated on FSc. Therefore, this BD FACSCalibur flow cytometer is capable of resolving beads down to 0.5 µm in size.

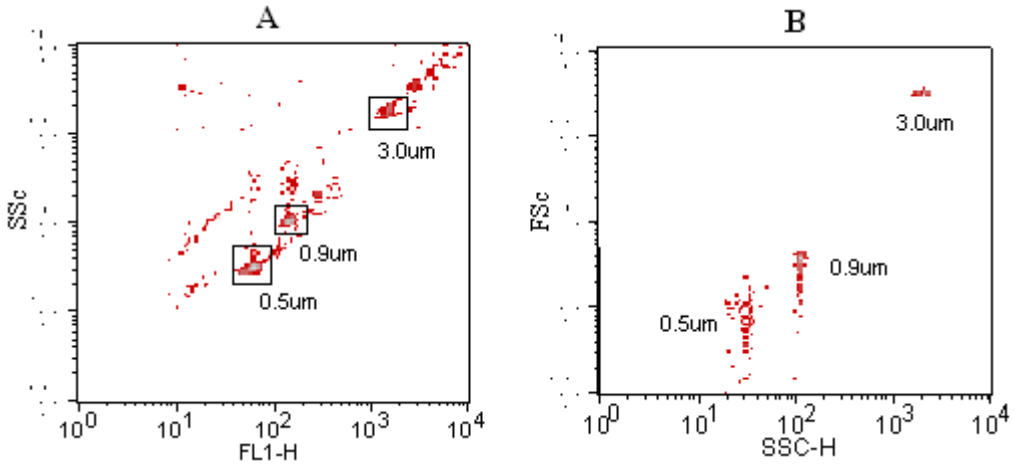

Figure 2.1 – Flow Cytometry plots of MegaMix Bead showing resolution of 0.5 µm and 0.9 µm beads. FSc – Forward Scatter, SSc – Side Scatter

2.3.1.2. Establishing an Microparticle Gate

In order to establish a MP gate, it was important to identify where platelets and PMPs appear on the FSc vs SSc plot. Calcium ionophore stimulation of PRP was used to generate PMPs from platelets. Gates were set so that in the PRP plot the major population falls in the platelet gate, and in the calcium ionophore stimulated PRP plot the major population falls in the MP gate (Figure 2.2). This gate was used to establish the MP gate for all future experiments.

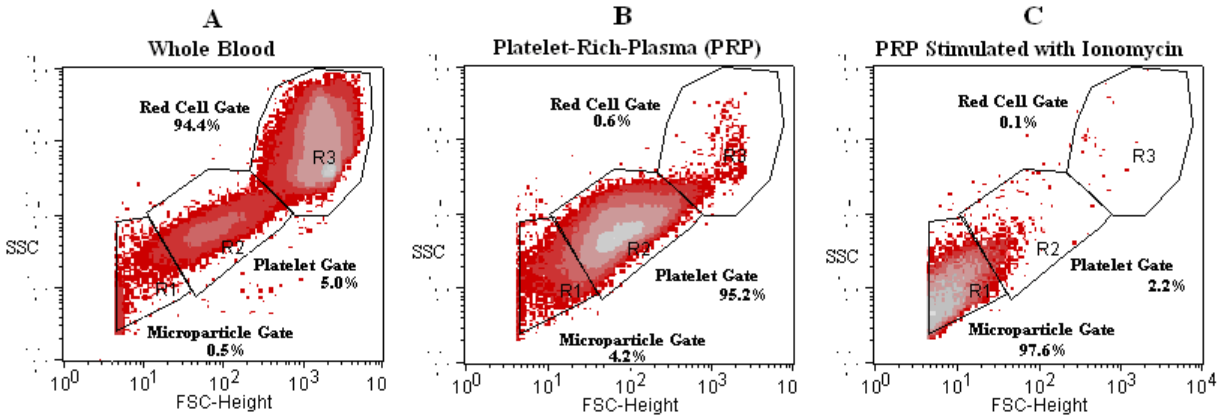

Figure 2.2 – SSC vs. FSC FACS plots showing distinct red cell, platelet and MP gates. Plot A shows analysis of unstimulated whole blood. Plot B shows analysis of unstimulated platelet-rich-plasma. There are few MPs and mainly platelets. Plot C shows analysis of platelet-rich-plasma stimulated with calcium ionophore for 5 hours. Most events now appear in the MP gate.

2.3.1.3. Populations of Microparticle Subtypes

Figure 2.3 shows example plots of different MP subtypes. Procoagulant MPs are identified by annexin V binding (A) and lactadherin binding (B), as they both bind to PS on the surface of procoagulant MPs. PMPs were identified by CD31+CD41+ markers (C). Several markers were trialed for the identification of EMPs; CD31+CD41- (C), CD144+ (D), CD62+ (E) and CD106+ (F). LMPs were identified by CD45+ marker (G). Granulocyte-derived MPs were identified by CD66B expression (H). Monocyte-derived MPs were identified by CD14 expression (I). T-cell-derived MPs were identified by CD3 (J). RBC-derived MPs were identified by glycophorin A (K). All plots are gated on the MP gate, and are not restricted to those that are annexin V+.

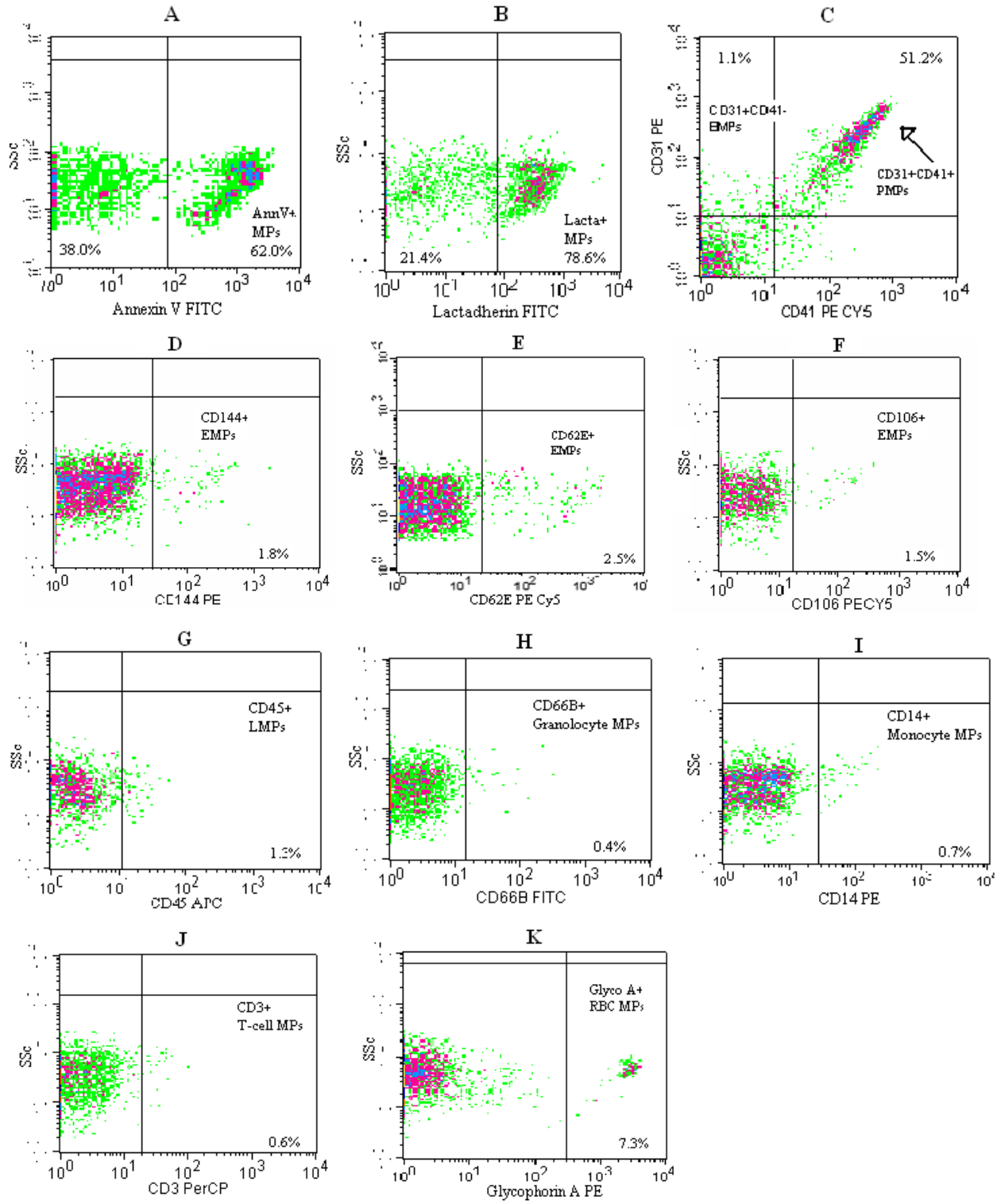

Figure 2.3 – Representative FACs plots of labelled PPP. A shows annexin V staining of MPs expressing PS. B shows lactadherin staining of MPs expressing PS. C shows CD31+CD41+ PMPs and CD31+CD41- EMPs. D, E and F show EMP staining. G shows CD45 staining of LMPs. H shows CD66B staining of granulocyte-derived MPs. I shows CD14 staining of monocyte-derived MPs. J shows CD3 staining of T-cell derived MPs. K shows glycophorin A staining of RBC MPs

It was originally thought that all MPs bind annexin V. However, now it is appreciated that some MPs do not bind annexin V, and that the proportion of MPs which bind annexin V may be dependent on the cellular origin of the MP. To investigate the proportion of annexin V positivity using the cell specific markers described above, a preliminary study was conducted, using 20 frozen samples from the OSA patients. OSA patients were used because they have higher levels of EMPs and LMPs than healthy controls.

Individual subtypes of MPs were assessed for their ability to bind annexin V. The percentage that bound annexin V in each MP subgroup varied greatly between individuals. The vast majority of PMPs and RBC-derived MPs were annexin V+. Other MP subtypes had a much lower percentage that bound annexin V, particularly CD31+CD41- EMPs and monocyte-derived MPs (Table 2.2).

Table 2.2 – Percentage of Microparticle Subtypes that Bound Annexin V

MP Subtype	% Annexin V+
CD31+CD41+ PMPs	93.4 % (±3.9 %)
CD31+CD41- EMPs	33.8 % (±14.8 %)
CD144+ EMPs	52.6 % (±18.9 %)
CD62E+ EMPs	70.4 % (±19.6 %)
CD106+ EMPs	62.0 % (±29.8 %)
CD45+ LMPs	56.8 % (±16.8 %)
CD66B+ Granulocyte MPs	N/A CD66B on same fluorochrome as AnnV
CD14+ Monocyte MPs	36.9 % (±18.1 %)
CD3+ T-cell MPs	45.3 % (±26.7 %)
Glycophorin A+ RBC MPs	95.9 % (±3.9 %)

Mean (±SD) percentage of MP subtype that bound annexin V in 20 OSA patients

2.3.1.4. Flow Rate calculated by Weighed Tube Method versus TruCount Tube Method

The flow rate of a flow cytometer can be calculated by either a weighed tube method or calibrated counting bead (TruCount tube) method. The comparison of flow rate calculation by the two methods showed that there was no significant difference. Therefore, the weighed tube method will be used throughout this study, as it does not require the purchase of additional consumables.

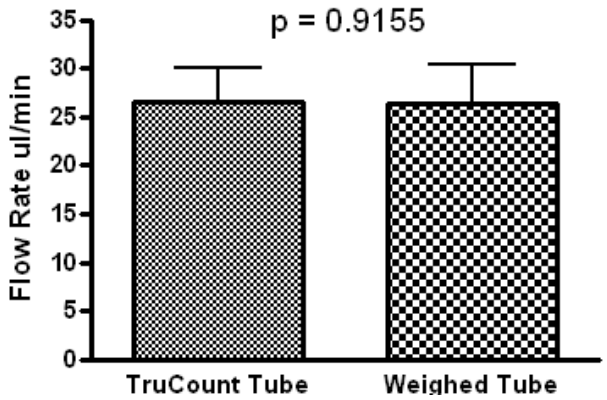

Figure 2.4 – Comparison of weighed tube and TruCount tubes for flow rate calculation. P-value shows paired T-test analysis of 10 pairs of results. Bar graph shows mean and SD.

2.3.2. Validation of the Assay

2.3.2.1. Linearity

It is important for this assay to be capable of measuring MPs at very high and very low concentrations, as levels can be very variable between individuals. Therefore, the linearity of each MP subgroup was assessed, to determine the assays ability to perform consistently at both high and low levels of MPs.

Table 2.3 – Table of Linearity Results for all of the Microparticle Subtypes

MP Subtype	R^2
Annexin V+ MPs	0.9999
Lactadherin+ MPs	0.9991
CD31+CD41+ PMPs	0.9992
CD31+CD41- EMPs	0.9321
CD144+ EMPs	0.9888
CD62E+ EMPs	0.9788
CD106+ EMPs	0.9976
CD66B+ Granulocyte MPs	0.9789
CD14+ Monocyte MPs	0.9637
CD45+ LMPs	0.9838
CD3+ T-cell MPs	0.9813
Glycophorin A+ RBC MPs	0.9952

The R^2 values are all close to 1.0000, showing that for all of the MP subtypes, results were closely correlated with the concentration of MPs added.

2.3.2.2. Intra and Inter-Assay Precision

The intra-assay precision indicates how consistently the assay performs within a single batch run of samples, and the inter-assay precision indicates how consistently the assay performs between different batch runs of samples. It is important for the assay to have good inter-assay precision, so that different batches of results can be compared to each other.

Table 2.4 – Table of Intra-assay and Inter-assay Precision

Mp Subtype	Intra-Assay CV%	Inter-Assay CV%
Annexin V+ MPs	6.2	23.0
Lactadherin+ MPs	6.2	25.9
CD31+CD41+ PMPs	6.7	26.7
CD31+CD41- EMPs	11.4	35.9
CD144+ EMPs	**27.3**	**56.0**
CD62E+ EMPs	17.9	26.0
CD106+ EMPs	16.9	36.8
CD45+ LMPs	19.9	37.5
CD66B+ Granulocyte MPs	17.2	28.5
CD14+ Monocyte MPs	**28.3**	33.2
CD3+ T-cell MPs	**26.0**	**84.1**
Glycophorin A+ RBC MPs	6.5	17.2

The majority of CV% values for the intra-assay precision are less than 20 %. However the intra-assay CV% values for CD144+ EMPs, CD14+ Monocyte MPs and CD3+ T-cell MPs were between 20 % and 30 %.

The CV% values for the inter-assay precision are less than 40 % for the majority of MP subtypes. However for CD144+ EMPs and CD3+ T-cell MPs the CV% values are very high, as even in OSA patients, levels of these MP subtypes are very low.

2.3.3. Sources of Variability within the Assay

2.3.3.1. Effect of Time before Centrifugation

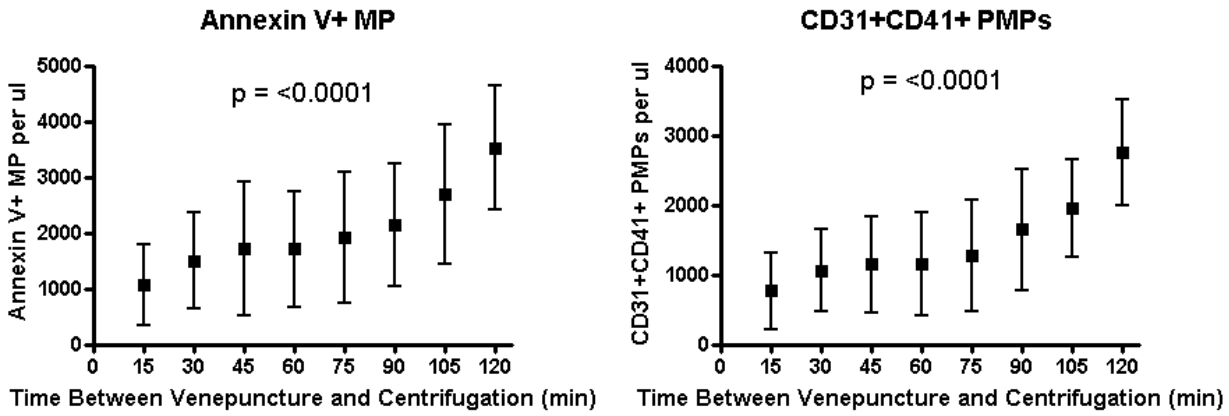

Figure 2.5 – Number of annexin V+ MPs and PMPs compared to the length of time before processing of blood samples. 8 citrate tubes were taken from controls and left for increasing length of time prior to centrifugation and freezing. Each sample was analysed 8 times and the mean for each sample was plotted. The bars show the mean and 95% CI for the eight controls at each time point (n=8)

The time between venepuncture and processing of samples has a significant impact on the numbers of MPs detected using this flow cytometric method. The number of annexin V+ MPs and PMPs increase as the length of time in which they are left before processing increases. There appears to be a plateau at around 1hr post venepuncture. Repeated measures ANOVA was significant for both annexin V+ MPs ($p < 0.0001$) and CD31+CD41+ PMPs ($p < 0.0001$), and the post hoc test revealed a significant linear trend over increasing time in both cases ($p < 0.05$). The level of MPs appear to more than double if blood samples are left for 2 hours before centrifugation and freezing, compared to processing immediately. Other MP subtypes were not examined.

2.3.3.2. Effect of Washing Platelet-Poor-Plasma before Staining

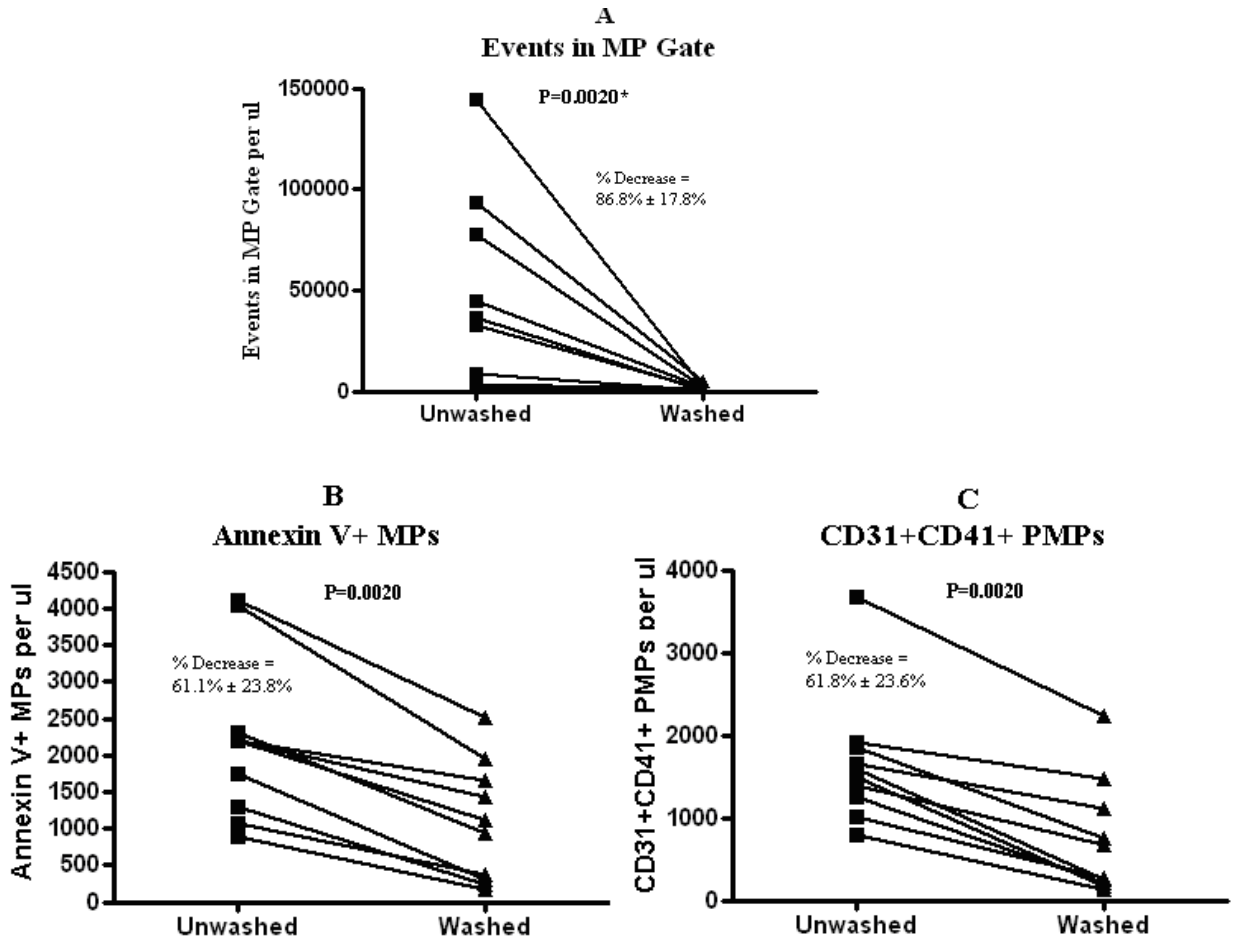

Figure 2.6 – Number of Events in MP Gate (A), Annexin V+ MPs (B) and CD31+CD41+ PMPs (C) for unwashed and washed samples. Unwashed samples were thawed and stained. Washed samples were thawed and centrifuged at 18,000 g for 30 min at RT, removing the supernatant, twice (n=10)

There was a significant decrease (p=0.002) in the number of events in the MP gate, particularly in samples with initially very high levels of MPs, following washing of PPP samples. There was also a significant decrease in annexin V+ MPs (p=0.002) and CD31+CD41+ PMPs (p=0.002) following washing of the plasma samples. There was no significant difference in the levels of CD31+CD41- EMPs, CD144+ EMPs, CD62E+ EMPs, CD106+ EMPs and CD45+ LMPs between washed and unwashed samples (data not shown).

2.3.3.3. Effect of Single versus Double Centrifugation

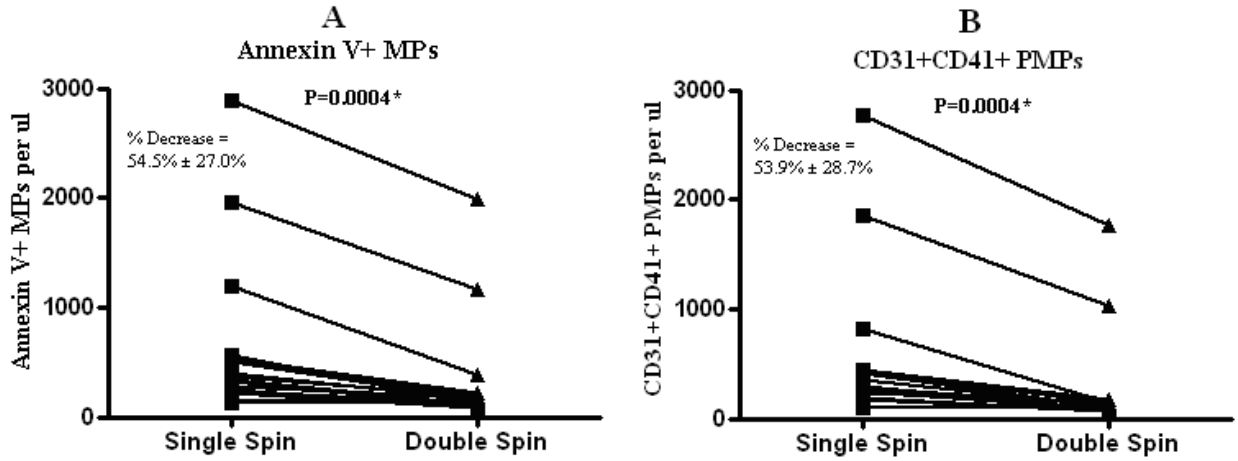

Figure 2.7 – Number of annexin V+ MPs (A) and CD31+CD41+ PMPs (B) for single and double centrifuged samples. Single spun samples were centrifuged at 1550 g for 20 min, before freezing at -80 ºC. Double spun samples were centrifuged at 1550 g for 20 min, followed by centrifugation at 13,000 g for 2 min, before freezing at -80 ºC (n=18)

The addition of a second spin at 13,000 g for 2 min, prior to freezing of platelet-poor-plasma significantly reduces the number of annexin V+ MPs (p=0.0004) and CD31+CD41+ PMPs (p=0.0004). The levels of CD31+CD41- EMPs, CD144+ EMPs, CD62E+ EMPs, CD106+ EMPs and CD45+ LMPs were not significantly different between single and double centrifuged samples (data not shown).

2.3.3.4. Fresh versus Frozen Samples

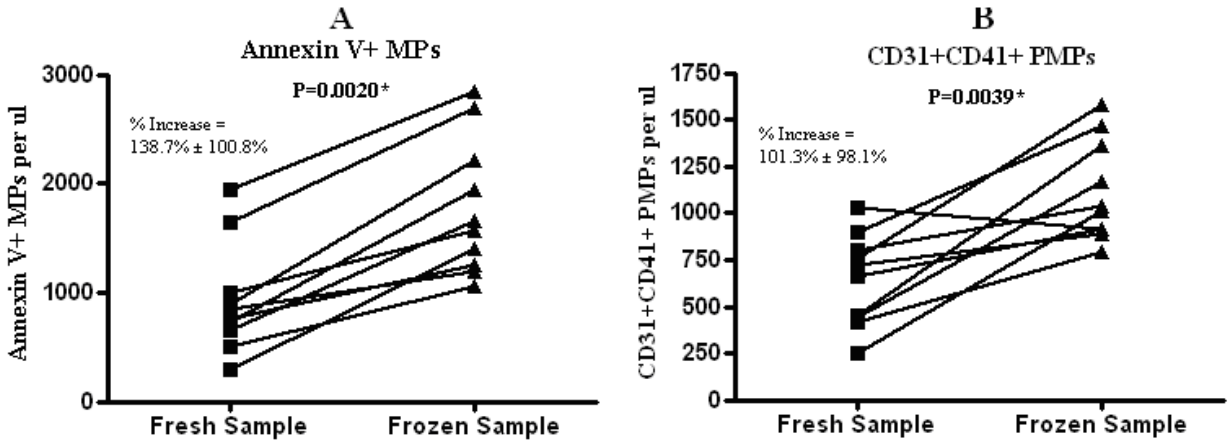

Figure 2.8 – Number of annexin V+ MPs (A) and CD31+CD41+ PMPs (B) for fresh and frozen samples, when using a double centrifugation protocol. Fresh samples were run within 3 hours of venepuncture. Frozen samples were centrifuged, frozen stored at -80 ºC for 1 month, then thawed and analysed (n=10)

Both annexin V+ MPs (p=0.0020) and CD31+CD41+ PMPs (p=0.0039) showed a significant increase in level, following freezing of the PPP sample. CD31+CD41- EMPs, CD144+ EMPs, CD62E+ EMPs, CD106+ EMPs and CD45+ LMPs did not show a significant change in level between fresh and frozen samples (data not shown).

2.3.3.5. Effect of Thawing Temperature on Microparticle Sample

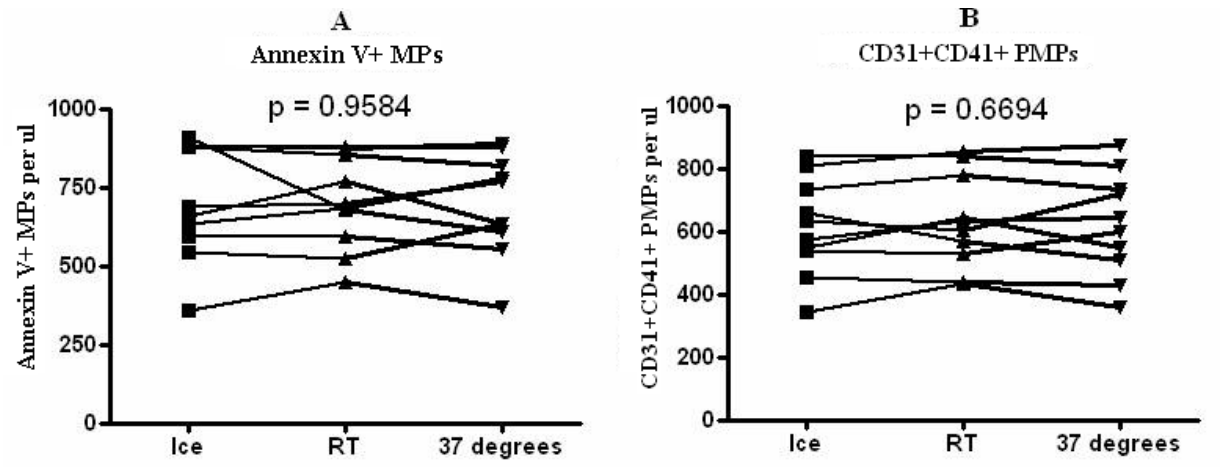

Figure 2.9 – Effect of thawing temperature on the numbers of annexin V+ MPs (A) and CD31+CD41+ PMPs (B) detected. Samples had been single centrifuged, 3 aliquots were taken and frozen at -80 ºC. For each control, one aliquot was thawed on ice, one at RT and one at 37 ºC (n=10)

There was no significant difference found in the number of annexin V+ MPs and CD31+CD41+ PMPs between samples that had been thawed on ice, at RT or at 37 ºC. Other MP subtypes were not assessed.

2.3.3.6. Effect of Long-term Storage at -80ºC of MP Samples

56 OSA patient samples were analysed for all MP subtypes firstly within 12 months of storage at -80 ºC, and then after an additional 20 months at -80 ºC.

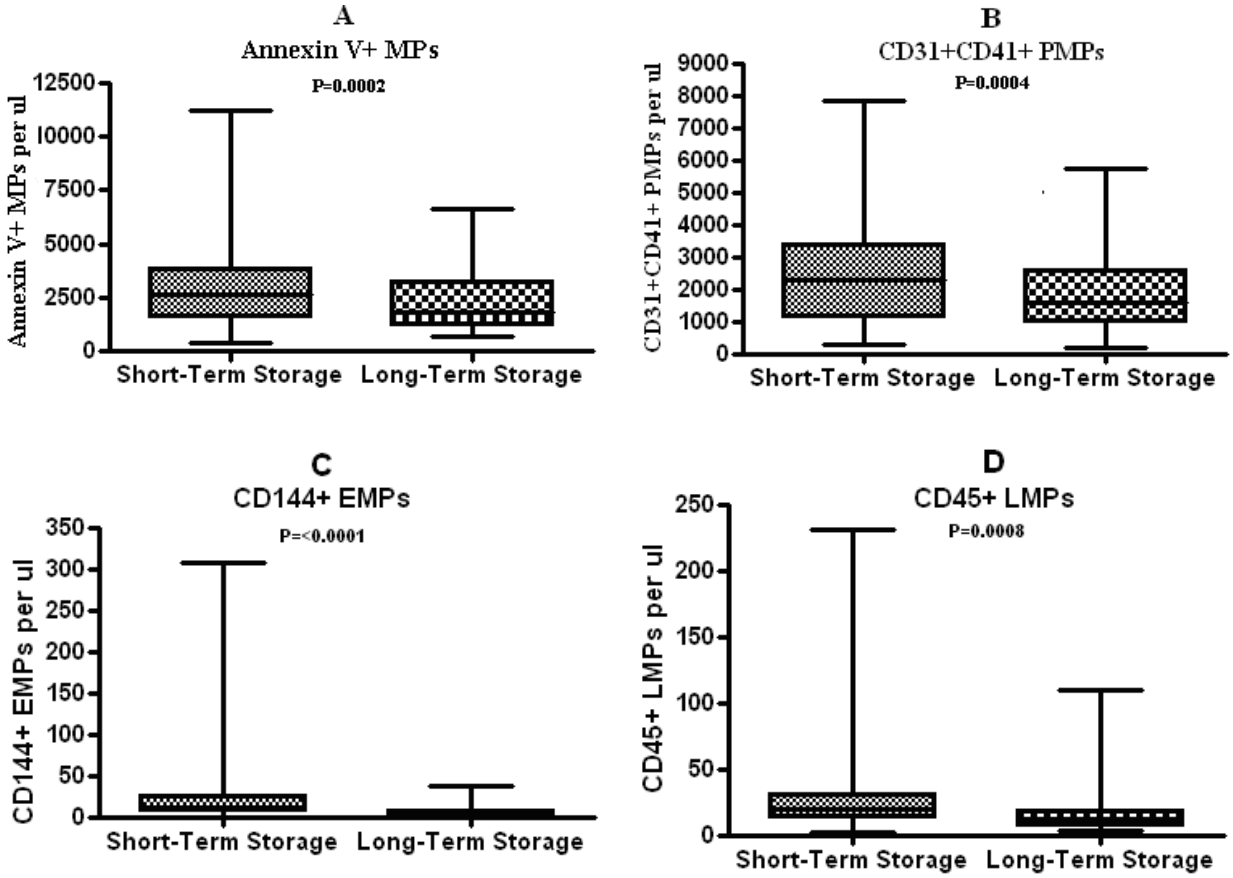

Figure 2.10 – Effects of long term storage on the numbers of annexin V+ MPs (A), CD31+CD41+ MPs (B), CD144+ EMPs (C) and CD45+ LMPs (D). The short-term storage samples were all processed within 12 months of freezing, the long-term storage samples were processed an additional 20 months later. Data presented as median with 95% CI. (n=56)

All subtypes of MPs were significantly reduced (annexin V+ MPs p=0.0002, CD31+CD41+ PMPs p=0.0004, CD144+ EMPs p<0.0001, CD106+ EMPs p=0.0021, CD45+ LMPs p=0.0008) after an additional 20 months storage, except for CD31+CD41- EMPs, which showed no significant change in levels (data not shown).

2.4. Discussion

This chapter aimed to develop a flow cytometric assay to measure subtypes of MPs, to establish the linearity, intra-assay precision and inter-assay precision for this assay, and to identify potential causes of variability in MP quantification. Validation showed that both the linearity and precision were comparable to other types of flow cytometry assays. This study found that minor protocol changes in MP analysis led to significant differences in MP levels.

2.4.1. Flow Cytometric Method for Detection of Microparticles

2.4.1.1. MegaMix Beads

MegaMix beads can be used to determine if an instrument is capable of resolving small particles. Most flow cytometers are optimised to detect cells, and many are unable to resolve these smaller particles. The MegaMix beads showed that the FACSCalibur used in this study is capable of resolving particles of 0.9 μm and 0.5 μm in size.

MegaMix beads can also be used to standardise settings between different instruments. The CV% values of PMP measurements between three different flow cytometric instruments standardised with MegaMix beads, was less than 12 % (Robert *et al.*, 2009). However, when MegaMix beads were used to set-up BD flow cytometers, PMP measurements were much more variable. It appears that the MegaMix strategy is more appropriate for instruments that measure FSc with a wide solid angle (1-19º) such as Beckman-Coulter, but were less suitable for instruments that measure FSc with a lower solid angle (1-8º) such as BD. The gating, defined by MegaMix beads, may not capture the whole PMP population on BD flow cytometers (Lacroix *et al.*, 2010). The use of calibrated beads provides a strategy for standardising flow cytometry analysis of MPs, but this appears be dependent on the instrument.

2.4.1.2. Establishing an Microparticle Gate

Calcium ionophore stimulation showed some overlap between platelets and PMPs on an FSc vs SSc plot (Figure 2.2), indicating that the MP gate may include some small platelets. This is to be expected as the smallest platelets form a continuum in size with the largest PMPs (Lynch and Ludlam, 2007). This is a potential limitation of detecting MPs by flow cytometry, which is difficult to control for.

2.4.1.3. Populations of Microparticle Subtypes

Although not positive for all MPs (Boulanger *et al.*, 2006; Connor *et al.*, 2010), annexin V is a useful marker to determine general levels of procoagulant MPs. Preliminary data suggests that different subtypes of MPs appear to bind annexin V at varying levels. For example, more than 90 % of PMPs and RBC MPs were annexin V+, whereas only around 50 % of CD144+ EMPs and LMPs were also annexin V+ (Table 2.2). Therefore, it is important to measure both annexin V positive and negative MPs, in order to not underestimate levels of some subtypes.

Lactadherin is another marker that binds PS, but it does not require the presence of calcium. It is potentially more sensitive at detecting PS, so could prove to be a useful marker of MPs (Dasgupta, 2006; Davizon and Lopez, 2009). Results from annexin V binding and lactadherin binding were found to be strongly positively correlated in this study (data not shown).

Bio-maleimide, a general MP marker, has been shown to give comparable results to annexin V. It is considerably cheaper and can be stored for longer, making it a useful marker for large scale analysis of total MP levels (Enjeti *et al.*, 2008). However, the use of bio-maleimide restricts the ability to measure other cell surface markers and, therefore, the cell of origin can not be determined. Phalloidin and Calcien actoxymethyl ester (calcien AM) have all been recently used to improve the detection of MPs. Phalloidin is a cyclic peptide which binds f-actin and calcien AM is a non-fluorescent marker that becomes fluorescent upon cleavage by cytosolic esterases and only stains intact MPs. These both avoid false positive MP counts from platelets or other cellular fragments (Yuana *et al.*, 2011).

Several subtypes of EMPs exist, and they are thought to be associated with different pathological mechanisms. For example, CD62E + EMPs are thought to be a marker of endothelial cell activation, while raised numbers of CD144+ and CD31+ EMPs may reflect structural damage of endothelial cells (Jimenez *et al.*, 2003b). CD144+ EMPs and CD31+CD41- EMPs have been shown to be correlated with haemodynamic severity of pulmonary hypertension, whereas CD62E+ EMPs did not correlate with severity, but were associated with CRP values in pulmonary hypertension patients (Amabile *et al.*, 2008). In addition, *in vitro* experiments have shown that different stimuli can lead to the release of phenotypically distinct EMPs. TNF-α stimulation results in elevation of CD62E+ EMPs, whereas CD31+EMPs were predominately produced during endothelial apoptosis. Therefore, phenotypic analysis of EMPs may prove to be useful in providing information reflecting the nature of endothelial injury (Jimenez *et al.*, 2003b).

In this study, CD31+CD41-, CD144, CD62E and CD106 were all explored as markers for EMPs. It is noted that the use of negative labelling may not be sufficiently specific to identify MP subtypes. CD31+CD41- and CD31+CD42- has been used previously to define EMPs (Chirinos *et al.*, 2005a; Periard *et al.*, 2007; Wang *et al.*, 2007a), however, it is appreciated that LMPs may also contribute to this population (Amabile and Boulanger, 2011).

2.4.1.4. Flow Rate calculated by Weighed Tube versus TruCount Tube Method

Comparison of these two methods for calculating flow rate of a flow cytometer showed no significant difference. Some studies use counting beads in every tube for microparticle analysis, which not only greatly increases the cost, but also introduces contaminating particles to the MP gate. Other studies use counting beads only to calculate the flow rate. The TruCount tubes utilised in this Chapter cost £4.50 per tube, which would significantly increase the overall cost of this assay. This would be an important consideration if MP analysis were to be offered as a routine clinical test in the future. Therefore, flow rate as calculated by the weighed tube method was used throughout the rest of this PhD, as it incurs no additional consumable costs and is comparable to calibrated counting beads.

2.4.2. Validation of the MP Assay

2.4.2.1. Linearity

The linearity results show that the values given for each of the MP subtypes correlated with the amount of plasma added to the assay (Table 2.3), suggesting that this assay is able to detect MPs present at variable levels in plasma samples, and the results obtained are independent of the concentration in the plasma. This is important characteristic of the assay, as MP levels can vary greatly between patients and controls.

2.4.2.2. Intra and Inter-Assay Precision

The intra-assay precision of most of the MP subtypes was good, as the CV% values were less than 20 %. This compares favourably with other types of flow cytometry assays. However, for the CD144+ EMPs, CD14+ Monocyte MPs and CD3+ T-cell MPs the CV% values were between 20-30 %, reflecting the fact that only very small populations of these MPs can be detected.

The inter-assay precision for the majority of MP subtypes was also good, with CV% values of less than 40 %. However, again for CD144+ EMPs and CD3+ T-cell MPs the CV% were much higher. This is due to the QC, used to establish inter-assay precision, having extremely low levels of these particular MP subtypes.

2.4.3. Sources of Variability within the Assay

2.4.3.1. Effect of Time before Centrifugation

The longer the time-delay between venepuncture and centrifugation, the higher the number of MPs detected. This is most likely due to platelets continuing to release MPs *ex vivo* in the blood tube. Therefore, it is very important that all samples in a study are collected and processed in the same way and within strict time-frames.

Often samples from a patient group will be taken in clinic, when it is not always possible to process the sample immediately. Healthy controls are often obtained in a laboratory environment, and are generally processed more quickly. This could result in significant differences between patient and control MP levels, simply due to the speed of processing.

2.4.3.2. Effect of Washing Platelet-Poor-Plasma before Staining

There was a significant decrease in the number of events in the MP gate, particularly in those with very high levels, following washing. This may be due to the loss of other particles which may be picked up in the MP gate, such as lipids. Chylomicrons are known to interfere in platelet analysis in flow cytometry (Cantero *et al.*, 1998) and they may also be picked up in the MP gate. There was also a significant decrease in annexin V+ MPs and CD31+CD41+ PMPs, following washing. It is likely that during washing, MPs stick together and therefore multiple vesicles are detected as a single event, artificially affecting the MP count. Alternatively, the centrifugation step of 18,000 g might not be sufficient to pellet all MPs. It would be necessary to check for MPs in the discarded supernatant, in order to determine this.

There was no significant difference in the levels of EMPs and LMPs before and after washing. A possible explanation could be that the washing process removes non-specific events from the MP gate, thereby enabling detection of smaller MP populations, such as EMPs and LMPs.

A recent study compared using a 2 min centrifugation pelleting step versus 20 min both at 17,000 g, and found no significant difference. This suggested that a shorter centrifugation time may be comparable and would be useful in a clinical setting (Dey-Hazra *et al.*, 2010).

2.4.3.3. Effect of Single versus Double Centrifugation

Of the published MP flow cytometric techniques, no consensus exists for the speed and timing of centrifugation. This study compared two commonly used centrifugation protocols (Jy *et al.*, 2004). The data here shows that a double centrifugation significantly decreases the number of annexin V+ MPs and CD31+CD41+ PMPs. This may be due to platelets remaining in the plasma following a single centrifugation, continuing to release MPs during the freeze/thaw process. A previous study found that an additional high-speed

centrifugation, reduces platelet counts in plasma by 65 % (van Ierssel *et al.*, 2010). However, Tesselaar *et al* found no platelet contamination following a single centrifugation at 1550 g for 20 minutes (Tesselaar *et al.*, 2007). The decrease in annexin V+ MPs and PMPs following a second centrifugation step could be due to the pelleting of some larger MPs at the higher centrifugation speed (Yuana *et al.*, 2011).

Not surprisingly levels of LMPs and EMPs were not significantly altered when using a single or double centrifugation, as any circulating leucocytes or rare endothelial progenitor cells would be removed by a single centrifugation at 1550 g.

There is currently no optimum centrifugation protocol recommended for the complete removal of platelets, without the loss of large MPs. Therefore, throughout this project, the centrifugation protocol used for each study was dependent on the facilities available, and consisted of either a single centrifugation at 1550 g for 20 min, or a double centrifugation at 1550 g for 20 min, followed by 13,000 g for 2 min. The centrifugation protocol was consistent within each study.

2.4.3.4. Fresh versus Frozen Samples

Even when using a double centrifugation, there was a significant increase in annexin V+ MPs and CD31+CD41+ PMPs following freezing of samples. It was previously thought that double centrifugation of 1550 g for 20 min and 13,000 g for 2 min should be sufficient to remove the majority of platelets from the sample, and therefore avoid the additional production of PMPs during freeze/thawing. However, a recent publication suggested that the double centrifugation protocol described in this chapter may still result in more residual platelets, than two centrifugations at 2500 g for 15 min (Stagnara *et al.*, 2012). The additional PMPs following freezing may be produced by any remaining platelets in the plasma (Mobarrez *et al.*, 2010; Trummer *et al.*, 2009), or by larger PMPs breaking down to produce smaller PMPs. However, the freezing of samples was shown not to affect the FSc vs SSc distribution of MPs (Amabile *et al.*, 2005), suggesting that the latter is unlikely. The samples in this study were not snap-frozen in liquid nitrogen, as recommended by the original protocol (Biro *et al.*, 2004), and this may have compounded the differences found between fresh and frozen samples.

Freezing of PPP samples is essential if MP measurement is to be feasible in large studies or for routine analysis. If researchers were restricted to the use of fresh samples for MP analysis, it would increase sample-to-sample variation and prevent laboratory collaborations.

2.4.3.5. Effect of Thawing Temperature on Microparticle Samples

Protocols for MP analysis recommend varying thawing temperatures. It was previously found that PMPs, were significantly reduced when thawed on ice, compared to those thawed at RT or at 37 ºC (Trummer *et al.*, 2009).

This study showed no statistically significant difference in the numbers of annexin V+ MPs and CD31+CD41+ PMPs in samples thawed at different temperatures. Other subpopulations of MPs were not analysed in this experiment. The lack of concordance between the previous study and this one may be due to differences in the methodology, such as the lack of snap-freezing.

2.4.3.6. Effect of Long-term Storage at -80ºC of Microparticle Samples

This study showed that most MP subtypes were significantly reduced by long term storage at -80 ºC. It is possible that MPs become smaller when frozen for a long time period, to a size below the threshold of our flow cytometer. The samples in this study were not centrifuged twice prior to freezing or snap frozen, which may have affected their stability. CD31+CD41- EMPs were not significantly affected by long-term storage, however, this may simply reflect their extremely low initial levels.

These finding were supported by Dey-Hazra *et al* (2010), who systematically measured MP levels after freezing. They found that annexin V+ MP levels initially increase following freezing for 14 days, but after 28 days the levels begin to decrease, and by 56 days they fall below the initial freshly analysed levels.

In large studies, blood samples are collected over a long time and MPs are analysed in batches. These results suggest that great care should be taken when analysing samples that have been stored at -80 ºC for variable time periods. Ideally all samples within a study would be analysed after storage at -80 ºC for an equal length of time.

2.4.3.7. Recommendations for Standardisation

The results obtained in this Chapter were published in Thrombosis Research (Ayers *et al.*, 2011) (Appendix 1). The following recommendations are made:

1) Blood samples should be processed within strict time-frames and sample handling should be identical between patient and control groups.
2) PPP samples can be washed prior to analysis, to remove non-specific particles, to concentrate samples, and to enable detection of smaller MP sub-groups. It should be noted that washing of samples may result in the loss of some MP populations.
3) A double centrifugation step can assist in the removal of platelets prior to freezing, to avoid additional PMP production. However, it may also result in the loss of some larger MPs. It is essential that the centrifugation protocol is consistent throughout a study.

4) Samples processed fresh and those frozen prior to analysis should not be directly compared.
5) If long-term storage of samples at -80 ºC is necessary, then ideally all samples should be stored for an equal length of time.

2.4.3.8. Microparticle Analysis Standardisation

Several groups are now working to improve the standardisation between laboratories measuring MPs. One obstacle is the lack of a reference sample for MP analysis. This would assist in the calibration of assays, and would also provide a basis for external quality control of assays. The Scientific and Standardisation Committee (SSC) Subcommittee on Vascular Biology are working on developing a reference sample for MPs to improve the consistency of MP analysis between laboratories (Freyssinet *et al.*, 2009).

Calibration beads, such as MegaMix, are one way to standardise the flow cytometry settings used for MP analysis. However, vesicles have a lower refractory index than polystyrene beads, so they scatter light about 10-fold less efficiently (Chandler *et al.*, 2011), and therefore cannot be used to replicate MPs when analysed by flow cytometry. Also their ability to standardise settings is machine dependent (Lacroix *et al.*, 2010).

A very recent study also examined the pre-analytical variables of MP analysis (Lacroix *et al.*, 2012). Investigation of time delay before processing samples showed that after 4 hours the levels were increased, supporting the data found in this study. Time delay only affected annexin V+ and PMPs, but not RBC MPs, suggesting that RBCs are not as quick to produce MPs following venepuncture. It was found that strong agitation of samples prior to centrifugation has an impact on MP levels, with annexin V+ and PMPs being sensitive (Lacroix *et al.*, 2012). This study was similar in design to the methods described in this Chapter, and it supports the majority of the findings.

The standardisation of pre-analytical variables for MP analysis is still an un-obtained goal. However, interest has grown in this area, with many recent publications. International conferences dedicated to MP and exosome analysis now provide a platform for discussions on such issues, and may resolve the debate in the near future.

2.4.4. Limitations

The high inter-assay CV% determined for CD144+ EMPs and CD3+ T cell-derived MPs was likely due to the very low levels of these particular subtypes in the sample. No samples with high levels of these MP subtypes were available in a large enough volume to conduct inter-assay analysis.

The experiments on sources of variability, except for the effect of long-term storage, were performed on healthy samples, with very low levels of EMPs and LMP subtypes. This may account for the lack of significant change between the pre-analytical variables in these subtypes of MPs. Ideally these experiments would be repeated using samples from patients with known elevations in EMPs and LMPs. However, in a clinical situation it would be difficult to obtain the multiple samples required.

For the work on sources of variability, only a limited panel of MP markers were used; annexin V, CD31, CD41, CD144, CD62E, CD106 and CD45. Ideally all markers, described in the methods, would have been used in these experiments.

2.4.5. Conclusions

A flow cytometric assay was adapted for use in this laboratory, and was successfully validated by linearity, intra-assay variation and inter-assay variation. Data presented in this Chapter shows that the levels of MP detected by flow cytometry are subject to change by several variables, such as time before processing, number of centrifugations, freezing of samples, and how long they stored at -80 ºC. It is essential to consider these factors when designing studies to measure MPs in different disease groups, to ensure that any differences in levels are due to the clinical condition, and not due to variations in the technique used to measure them.

A consensus on the methodological approach to MP analysis and the standardisation of techniques will be essential for successful development of MP technologies, allowing direct comparison of results between studies and leading to a greater understanding of MPs in disease.

Chapter 3 – *In vitro* Generation of Microparticles

3.1. Introduction

The cell specific markers described in Chapter 2 have been well described for their use in cells and platelets. However, as it is known that the antigenic phenotype of MPs varies from their cell of origin (Abid Hussein *et al.*, 2003), it was important to validate these markers on MPs. The cell-specific markers chosen have all been used to identify cell-specific MPs in previous studies. Some subtypes of MPs are only present in very small proportions in the circulation, even in disease. Therefore it was useful to generate these MPs *in vitro* to titrate the concentration of antibody needed and to confirm its suitability for MP analysis.

3.1.1. Stimulation of Circulating Cells to Produce Microparticles *In Vitro*

3.1.1.1. Stimulation of Platelets to Produce Platelet-Derived MPs *In Vitro*

It has been well established that PMPs can be generated from platelets by incubating platelet-rich-plasma (PRP) with calcium ionophore A23187 (Nieuwland and Sturk, 2007; Yin *et al.*, 2008), and this was demonstrated in Chapter 2 (Figure 2.1), where platelet stimulation was used to define the MP gate. The resulting PMPs produced have been previously found to express CD31, CD42b, CD61 and CD62P on their surface (Abid Hussein *et al.*, 2003).

3.1.1.2. Stimulation of Leucocytes to Produce Leucocyte-Derived MPs *In Vitro*

The generation of neutrophil-derived MPs, which express CD66B, was demonstrated by priming isolated neutrophils with TNF-α, followed by stimulation with anti-neutrophil cytoplasmic antibodies (ANCAs) (Hong *et al.*, 2012).

Monocyte-derived MPs can be generated from a monocytic cell line, stimulated with lipopolysaccharide (LPS). These monocyte MPs contain IL-1β and may amplify inflammation, by enhancing the activation of endothelial cells (Wang *et al.*, 2011).

T cell-derived MPs have been generated from a human T cell line stimulated for 6 hours with phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA). These MPs are capable of inducing the production of TNF and IL-1β from cultured human monocytes, suggesting that they express similar monocyte-activating factors, as T cells (Scanu *et al.*, 2008).

3.1.1.3. Stimulation of RBCs to produce RBC-derived MPs *In Vitro*

RBCs incubated with calcium ionophore, have been shown to release RBC-derived MPs, which express glycophorin A and bind to annexin V (Nantakomol *et al.*, 2011). RBC-derived MPs are also increasingly produced during the storage of RBC concentrates (Rubin *et al.*, 2008).

3.1.1.4. Stimulation of Endothelial Cells to Produce Endothelial Cell-Derived MPs *In Vitro*

Endothelial cells are difficult to isolate from humans, unlike platelets, leucocytes and RBCs. In order to generate EMPs *in vitro* it was necessary to culture endothelial cells. The most common endothelial cell culture used for this purpose are Human Umbilical Vein Endothelial Cells (HUVECs). Stimulation of HUVECs with several agonists, such as TNF-α (Combes *et al.*, 1999), IL-1α (Abid Hussein *et al.*, 2003) and CRP (Wang *et al.*, 2007b) have been shown to cause the release of EMPs. In this Chapter, TNF-α, IL-1α, CRP and IL-6 are used to stimulate the release of EMPs from HUVECs.

TNF-α is a proinflammatory cytokine which can induce both cell proliferation and cell apoptosis. It has previously been shown to trigger the release of EMPs from HUVECs (Wang *et al.*, 2005). IL-1α is also a proinflammatory cytokine released by many cell types, and it can cause activation of endothelial cells. IL-1α has been used to induce HUVECs to produce EMPs (Abid Hussein *et al.*, 2003), and *in vivo* its levels are positively correlated with levels of CD144+ EMPs (Tramontano *et al.*, 2010). IL-6 can act as both a proinflammatory cytokine, by triggering the acute phase response, and an anti-inflammatory cytokine, by inhibiting TNF-α and IL-1. Levels of IL-6 were found to be positively correlated with levels of CD31+ EMPs in healthy individuals (Chirinos *et al.*, 2005b). CRP is an acute phase protein, triggered by IL-6, which can opsonise dying cells and trigger the classical complement pathway. CRP has been shown to trigger the release of EMPs from human aortic endothelial cells *in vitro* (Devaraj *et al.*, 2011).

3.1.2. Aims of this Chapter

1) To stimulate platelets, RBCs and leucocytes to produce MPs, and to use these MPs to confirm the suitability of markers for MP analysis.

2) To stimulate HUVECs with a range of agonists, to confirm the suitability of endothelial markers for EMP analysis, and to determine if different stimuli lead to the release of phenotypically distinct EMPs.

3.2. Methods

3.2.1. Stimulation of Circulating Cells to Produce Microparticles *In Vitro*

3.2.1.1. Stimulation of Platelets to Produce Platelet-Derived Microparticles *In Vitro*

Citrate blood was taken from 3 healthy controls (Oxford REC No: 11/SC/0183) and PRP was obtained by centrifugation at 200 g for 10 min. 100µl of PRP was stimulated with 0.625 µg/ml, 1.25 µg/ml or 2.5 µg/ml calcium ionophore A23187 for 2 hours at 37 ºC. 10 ul of un-stimulated PRP or stimulated PRP was labelled for PMPs with annexin V-FITC, CD31-PE and CD41-PE Cy5, for 30 min and then acquired by flow cytometry.

3.2.1.2. Stimulation of Leucocytes to Produce Leucocyte-Derived Microparticles *In Vitro*

Citrate blood was taken from 3 healthy controls and whole blood was diluted 1:1 with PBS-Ca. 200 µl of diluted blood was stimulated with 0.25 µg/ml PMA (Sigma, Kent, UK) + 0.625 µg/ml calcium ionophore (Stim 1), with 0.75 µg/ml PMA + 1.25 µg/ml calcium ionophore (Stim 2), or with 1.5 µg/ml PMA + 2.5 µg/ml calcium ionophore (Stim 3), for 2 hours at 37 ºC. Following incubation, samples were centrifuged at 18,000 g for 30 min, supernatant was removed and replaced with PBS-Ca. 10 µl of unstimulated whole blood or stimulated whole blood was stained for LMPs, granulocyte-derived MPs and monocyte-derived MPs with CD45-APC, CD66B-FITC and CD14-PE, for 30 min, and then acquired by flow cytometry.

3.2.1.3. Stimulation of RBCs to produce RBC-derived Microparticles *In Vitro*

The stimulation protocol, described above for leucocytes, was used to stimulate RBCs in whole blood. 10 µl of un-stimulated or stimulated whole blood was stained for RBC-derived MPs with glycolphorin A-PE, for 30 min, and then acquired by flow cytometry.

3.2.2. Stimulation of HUVECs to Produce Endothelial Cell-Derived Microparticles *In Vitro*

3.2.2.1. HUVEC Culture

HUVECs (Health Protection Agency (HPA) Culture Collections, UK) were thawed at 37 ºC and grown in Endothelial Cell Growth Media (HPA Culture Collections, UK) in T75 flasks (Sarstedt, Leicester, UK) at 37 ºC, 5.0 % CO_2 in a Hera Cell 240 Incubator (ThermoScientific, Hampshire, UK). Media was changed every other day, and once confluent, cells were split 1:2. Cells were detached from the flask by vigorous pipetting to avoid the use of Trypsin. Confluent cells were split and transferred to a 48-well plate (BD, UK). Media was changed every other day. HUVECs were used for stimulation when 90 % confluent.

3.2.2.2. Stimulation of HUVECs

Media was removed from the wells and cells were washed twice with 500 µl of Hank's Balanced Salt Solution (HBSS) (HPA Culture Collections, UK). 500 µl of agonist concentration was added to the wells, as listed in Table 3.1:

Table 3.1 - Agonists used for HUVEC Stimulation

Stimulant	Company	Concentration
Media Alone		
TNF-α	Enzo Life Sciences, Exeter, UK	10 ng/ml
TNF-α	Enzo Life Sciences, Exeter, UK	100 ng/ml
TNF-α	Enzo Life Sciences, Exeter, UK	1000 ng/ml
IL-1α	Sigma-Aldrich, Kent, UK	5 ng/ml
IL-1α	Sigma-Aldrich, Kent, UK	50 ng/ml
CRP	Sigma-Aldrich, Kent, UK	20 µg/ml
CRP	Sigma-Aldrich, Kent, UK	200 µg/ml
IL-6	eBioscience, Hatfield, UK	1 ng/ml

At 1 hour, 5 hours and 25 hours following stimulation with the above agonists, 100 µl of cell supernatant was removed, and replaced with 100 µl of the same agonist concentration. At each time point, the supernatant was centrifuged at 150 g for 5 min to pellet and remove any cells. 80 µl of supernatant was frozen at -80 ºC, until MP analysis was performed.

3.2.2.3. Analysis of Endothelial Cell-Derived Microparticles from Stimulated HUVECs

EMPs were assessed in the HUVEC supernatants by flow cytometry. 20 µl of supernatant was added to the appropriate antibody cocktail in 50 µl PBC-Ca. EMPs were stained with annexin V-FITC, lactadherin-FITC, CD31-PE, CD62E-PE Cy5,CD106-PE Cy5 and CD144-PE. Samples were acquired according to the method described in Chapter 2. EMP measurement was carried out in three staining experiments.

3.2.3. Statistics

Statistics were performed using GraphPad Prism 5 (GraphPad Software, San Diego). Effect of stimulation on levels of PMPs, LMPs and RBC MPs were determined by paired T-tests between un-stimulated and stimulated samples. Effect of stimulation of HUVECs on levels of EMPs after 25 hours was determined by a one-way ANOVA followed by Bonferroni's Multiple Comparison test. A p-value of <0.05 was considered statistically significant.

3.3. Results

3.3.1. Stimulation of Circulating Cells to Produce Microparticles *in vitro*

3.3.1.1. Stimulation of Platelets to Produce Platelet-Derived Microparticles *In Vitro*

To confirm that *in vitro* generated PMPs bind to the annexin V, CD31 and CD41 markers, PRP was stimulated with calcium ionophore to generate PMPs.

Stimulation of PRP with all three concentrations caused a trend to increase in PMPs, with 2.5 µg/ml calcium ionophore (Stim 3) causing a significant increase in annexin V+ PMPs (Figure 3.1A), CD31+ PMPs (Figure 3.1B) and CD41+ PMPs (Figure 3.1C).

Conversely, stimulation of PRP with calcium ionophore at all three concentrations, led to a significant reduction in CD31+ and CD41+ platelets (Figure 3.1D and 3.1E).

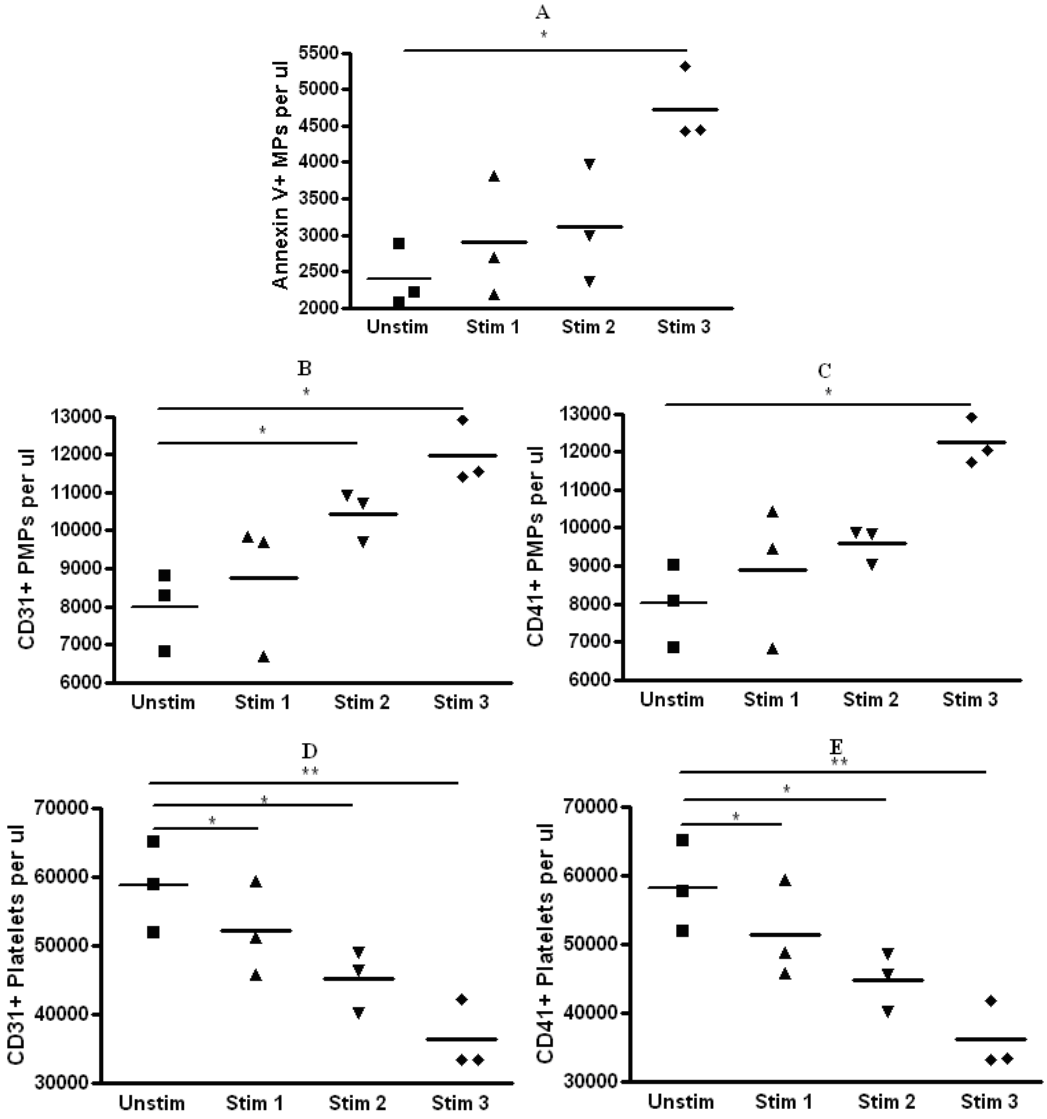

Figure 3.1 - Effect of stimulation on levels of annexin V+ MPs (A), CD31+ PMPs (B), CD41+ PMPs (C), CD31+ platelets (D) and CD41+ platelets (E). Paired-T-test performed (* $p<0.05$, ** $p<0.01$). Platelets were incubated with 0.625 µg/ml (Stim 1), 1.25 µg/ml (Stim 2) and 2.5 µg/ml (Stim 3) calcium ionophore for 2 hours at 37 ºC

3.3.1.2. Stimulation of Leucocytes to Produce Leucocyte-Derived Microparticles *In Vitro*

To confirm that *in vitro* generated MPs bind to the CD45, CD66B and CD14 markers, whole blood was stimulated with PMA and calcium ionophore to generate LMP release.

Stimulation with 0.25 µg/ml PMA + 0.625 µg/ml calcium ionophore (Stim 1) caused a trend for CD45+ LMPs and CD14+ monocyte-derived MPs to rise, and a significant increase in CD66B+ granulocyte-derived MPs. Stimulation with 0.75 µg/ml PMA + 1.25 µg/ml calcium ionophore (Stim 2) caused a significant increase in CD45+ LMPs (Figure 3.2A), CD14+ monocyte-derived MPs (Figure 3.2B) and CD66B+ granulocyte-derived MPs (Figure 3.2C), compared to non-stimulated cells. There appears to be a drop-off in CD45+ LMP and CD66B+ granulocyte MP production at the highest agonist concentration (Stim 3). This could be due to this very high concentration causing cells to die by necrosis, instead of being activated or dying by apoptosis.

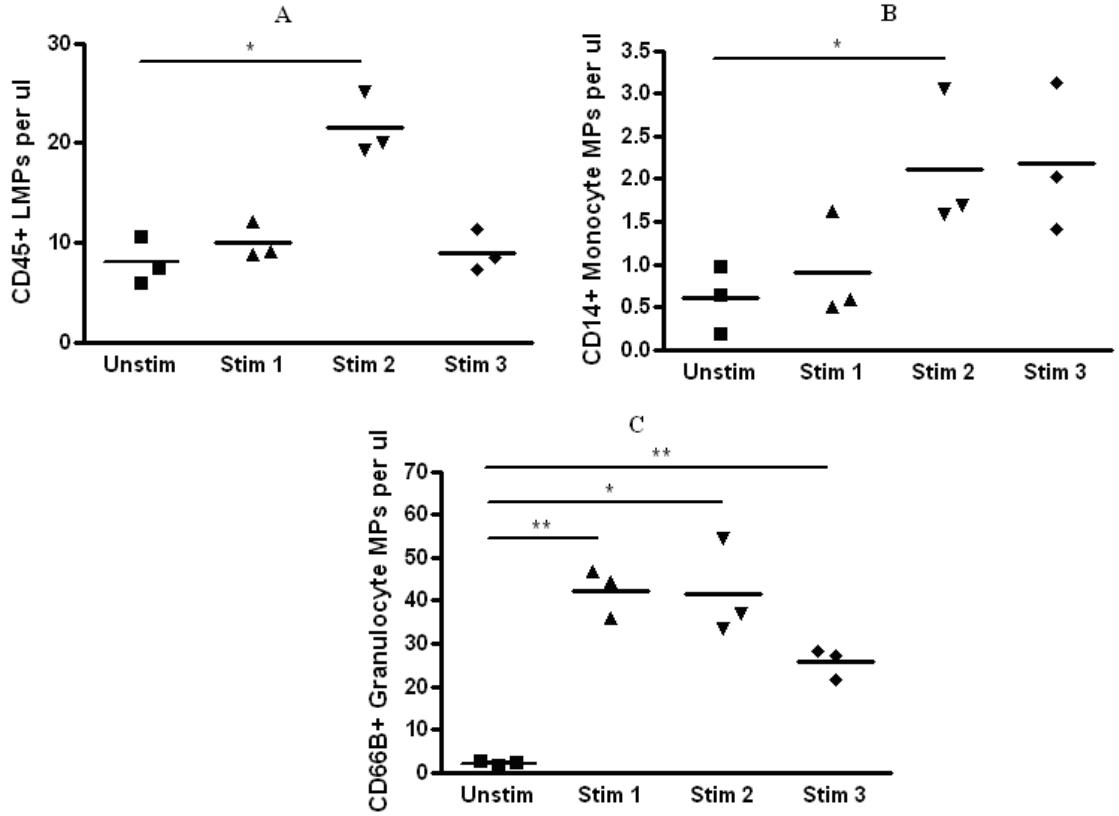

Figure 3.2 - Effect of stimulation on levels of CD45+ LMPs (A), CD14+ monocyte-derived MPs (B), and CD66B+ granulocyte-derived MPs (C). Paired-T-test performed (* $p<0.05$, ** $p<0.01$). Whole blood was stimulated with 0.25 µg/ml PMA + 0.625 µg/ml calcium ionophore (Stim 1), with 0.75 µg/ml PMA + 1.25 µg/ml calcium ionophore (Stim 2), or with 1.5 µg/ml PMA + 2.5µg/ml calcium ionophore (Stim 3), for 2 hours at 37 ºC

3.3.1.3. Stimulation of RBCs to produce RBC-derived Microparticles *In Vitro*

To confirm that *in vitro* generated MPs bind to glycophorin A, whole blood was stimulated with PMA and calcium ionophore to generate RBC-derived MPs.

Stimulation of whole blood with 1.5 µg/ml PMA + 2.5 µg/ml calcium ionophore (Stim 3) caused a significant increase in RBC-derived MPs (Figure 3.3A), and led to a significant decrease in the number of glycophorin A+ cells detected (Figure 3.3B).

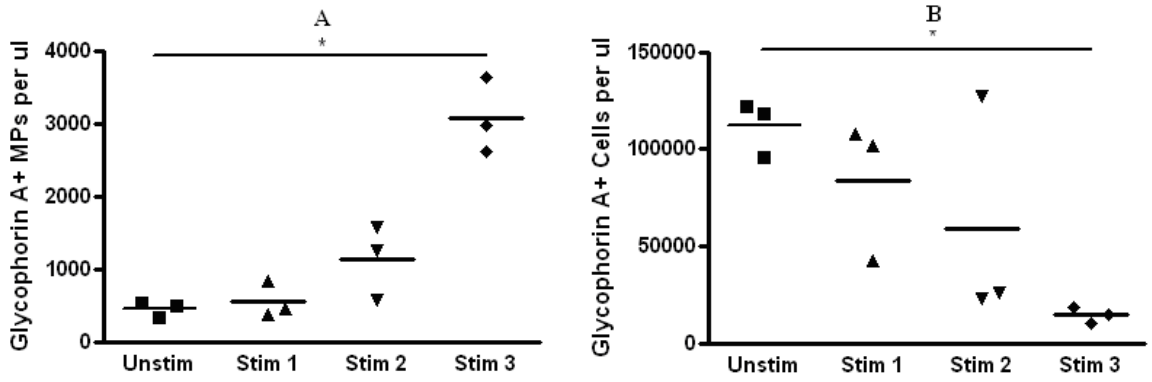

Figure 3.3 - Effect of stimulation on levels of glycophorin A+ RBC-derived MPs (A) and glycophorin A+ RBCs (B). Paired-T-test performed (* $p<0.05$). Whole blood stimulated with 0.25 µg/ml PMA + 0.625 µg/ml calcium ionophore (Stim 1), with 0.75 µg/ml PMA + 1.25 µg/ml calcium ionophore (Stim 2), or with 1.5 µg/ml PMA + 2.5 µg/ml calcium ionophore (Stim 3), for 2 hours at 37 ºC.

3.3.2. Stimulation of HUVECs to Produce Endothelial Cell-Derived Microparticles *In Vitro*

TNF-α, IL-1α, CRP and IL-6 were chosen as agonists to stimulate HUVECs, as they have been shown in previous studies to successfully generate the release of EMPs (Abid Hussein *et al.*, 2003; Devaraj *et al.*, 2011; Dignat-George and Boulanger, 2011; Wang *et al.*, 2005). They were also chosen because they are physiological agonists, and may be responsible for triggering EMP release *in vivo*.

EMPs in the supernatant were labelled with the EMP markers described in Chapter 2, to identify an increase in levels following stimulation. Statistics were performed on EMP levels at 25 hours following stimulation, as this was identified as the time point when the greatest increase in levels was detectable. This is in agreement with Jimenez *et al*, who found that 24 hours incubation led to maximal EMP release from cultured endothelial cells (Jimenez *et al.*, 2003b).

3.3.2.1. TNF-α Stimulation of HUVECs

TNF-α at all three concentrations significantly increased annexin V+ EMPs ($p<0.001$, Figure 3.4A), lactadherin+ EMPs ($p<0.001$, Figure 3.4B), CD31+ EMPs ($p<0.001$, Figure 3.4C) and CD62E+ EMPs ($p<0.001$, Figure 3.4D), compared to media alone. TNF-α stimulation at 100 ng/ml also significantly increased CD106+ EMPs ($p<0.01$, Figure 3.4E), and at 1000 ng/ml significantly increased CD144+ EMPs ($p<0.01$, Figure 3.4F), compared to media alone.

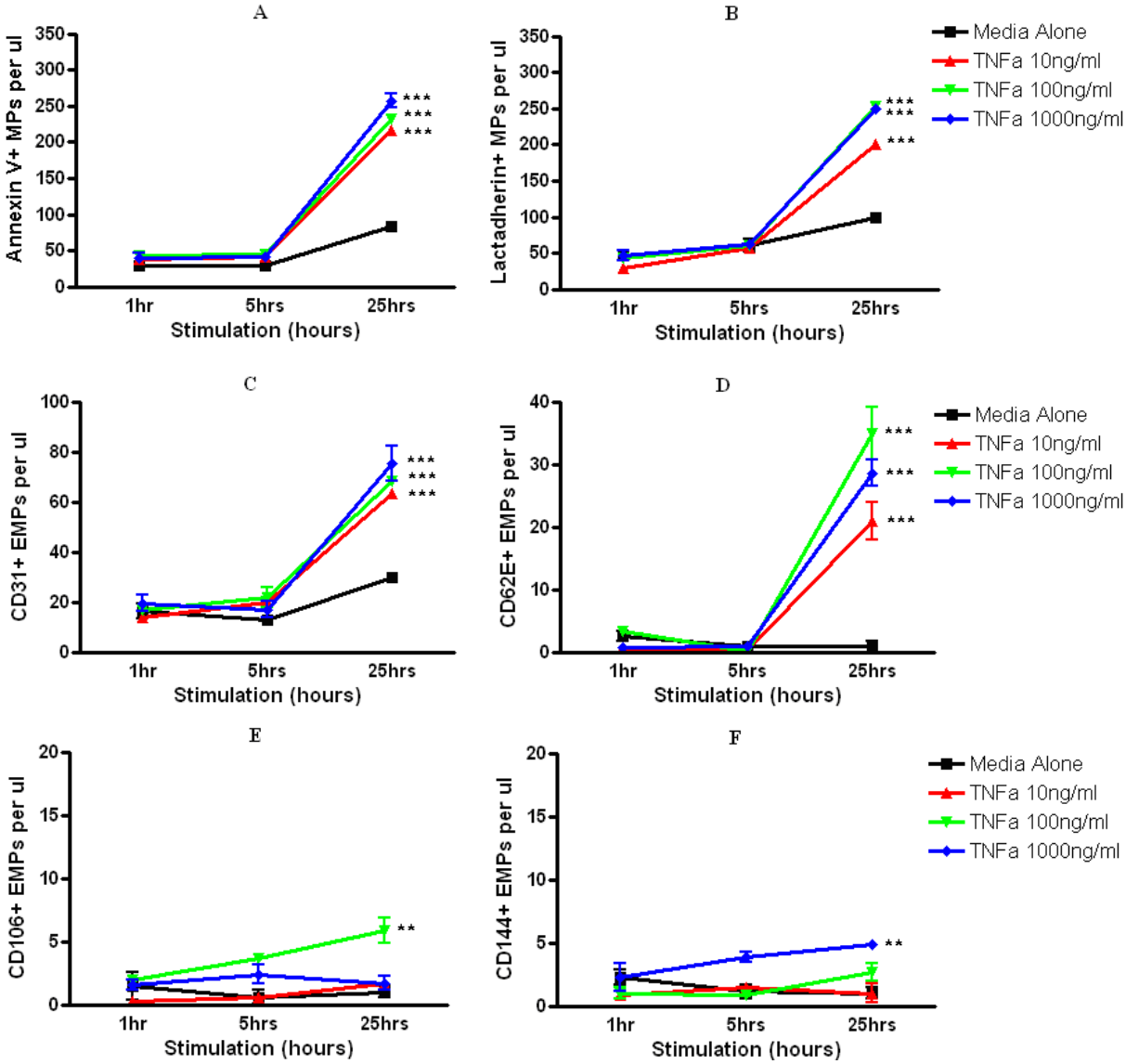

Figure 3.4 - Effect of TNF-α stimulation on HUVECs release of annexin V+ EMPs (A), lactadherin+ EMPs (B), CD31+ EMPs (C), CD62E+ EMPs (D), CD106+ EMPs (E) and CD144+ EMPs (F) after 25 hours. Points show mean and SEM of three staining experiments (**p<0.01, ***p<0.001).

3.3.2.2. IL-1α Stimulation of HUVECs

Stimulation of HUVECs with IL-1α at 5 ng/ml significantly increased annexin V+ EMPs ($p<0.001$, Figure 3.5A), lactadherin+ EMPs ($p<0.001$, Figure 3.5B), CD31+ EMPs ($p<0.001$, Figure 3.5C) and CD62E+ EMPs ($p<0.001$ Figure 3.5D), compared to media alone.

Stimulation of HUVECs with IL-1α at 50 ng/ml significantly increased annexin V+ EMPs ($p<0.05$, Figure 3.5A), lactadherin+ EMPs ($p<0.01$, Figure 3.5B), CD62E+ EMPs ($p<0.001$, Figure 3.5D) and CD144+ EMPs ($p<0.01$, Figure 3.5F), compared to media alone.

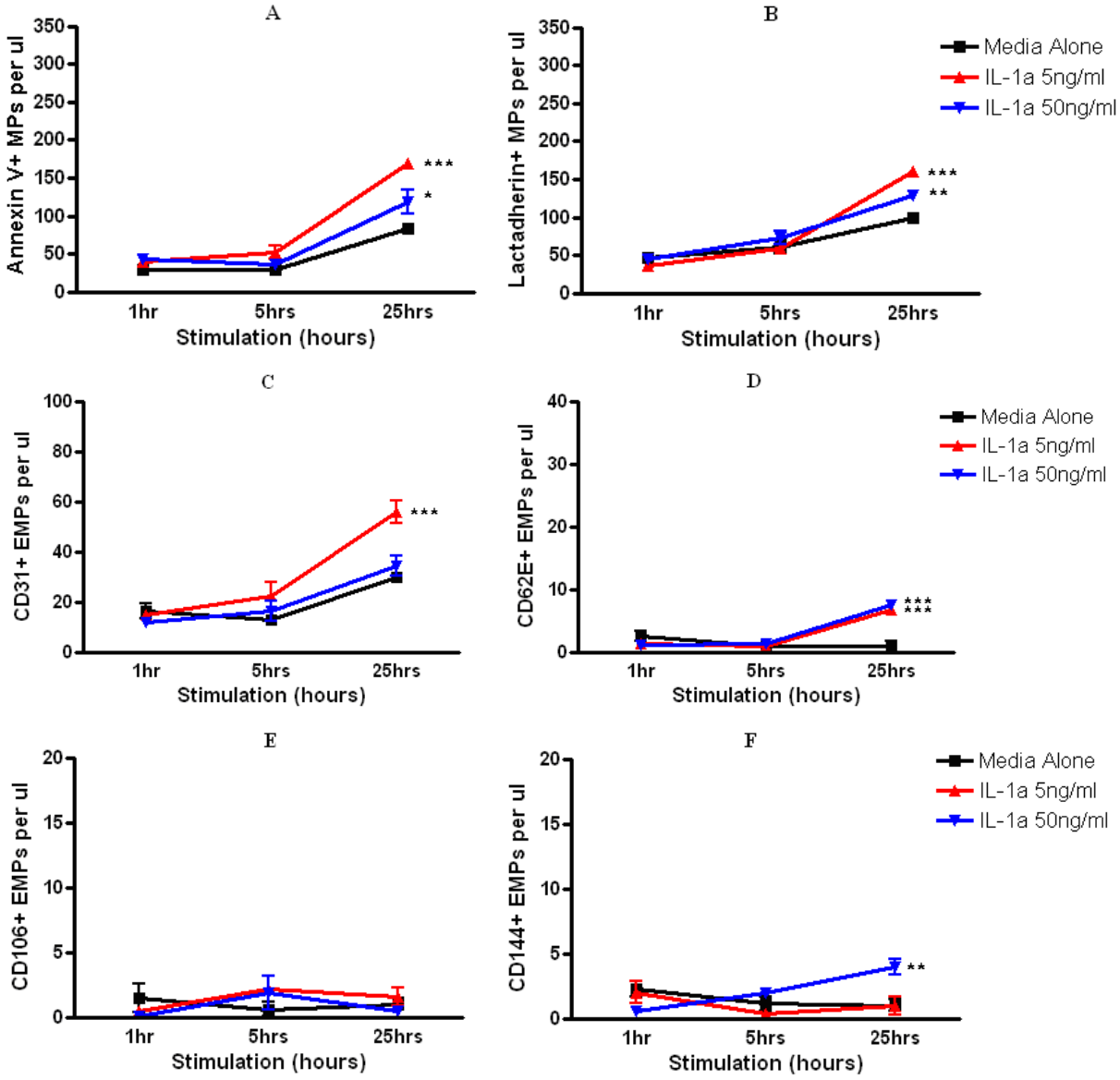

Figure 3.5 - Effect of IL-1α stimulation on HUVECs release of annexin V+ EMPs (A), lactadherin+ EMPs (B), CD31+ EMPs (C), CD62E+ EMPs (D), CD106+ EMPs (E) and CD144+ EMPs (F) after 25 hrous. Points show mean and SEM of three staining experiments (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

3.3.2.3. CRP and IL-6 Stimulation of HUVECs

Stimulation of HUVECs with CRP at 20 µg/ml and at 200 µg/ml significantly increased the number of CD31+ EMPs ($p<0.001$, Figure 3.6C), compared to media alone. No other EMP subtype was significantly increased by CRP stimulation (Figure 3.6).

Stimulation of HUVECs with IL-6 at 1 ng/ml caused a significant increase in annexin V+ EMPs ($p<0.01$, Figure 3.6A), lactadherin+ EMPs ($p<0.001$, Figure 3.6B), CD31+ EMPs ($p<0.001$, Figure 3.6C) and CD144+ EMPs ($p<0.05$, Figure 3.6F), compared to media alone.

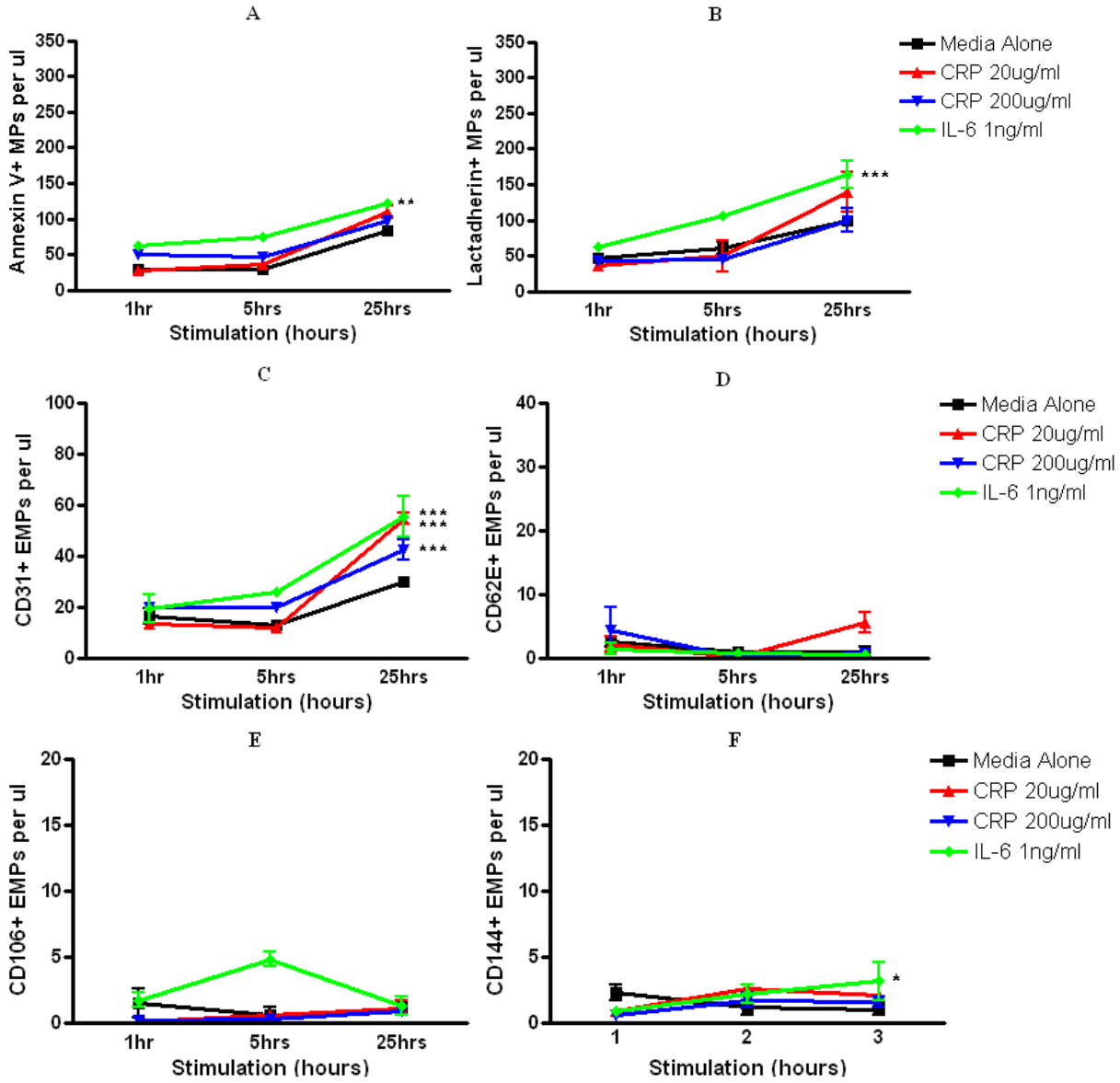

Figure 3.6 - Effect of CRP and IL-6 stimulation on HUVECs release of annexin V+ EMPs (A), lactadherin+ EMPs (B), CD31+ EMPs (C), CD62E+ EMPs (D), CD106+ EMPs (E) and CD144+ EMPs (F) after 25 hours. Points show mean and SEM of three staining (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

3.4. Discussion

3.4.1. Stimulation of Circulating Cells to Produce Microparticles *In Vitro*

3.4.1.1. Stimulation of Platelets to Produce Platelet-derived Microparticles *In Vitro*

Calcium ionophore is thought to directly facilitate the transport of calcium ions across the plasma membrane, and can cause cell activation and proliferation (Dedkova *et al.*, 2000). In this Chapter, stimulation of PRP with calcium ionophore, for 2 hours at 37 ºC, led to a significant increase in annexin V+ PMPs, CD31+ PMPs and CD41+ PMPs (Figure 3.1A – 3.1C) and a significant reduction in CD31+ and CD41+ platelets (Figure 3.1D – 3.1E). The increase in PMP levels was not as high as expected from the subsequent loss of CD31+ and CD41+ platelets, or as that seen in Chapter 2 (Figure 2). It may be that a longer incubation time, at a lower agonist concentration (as used in Chapter 2), would result in a more dramatic increase in MPs.

CD31 and CD41 are well-characterised for their expression on platelets, and this shows that they are also expressed on the MPs derived from platelets *in vitro*. The binding of annexin V to PMPs indicates their expression of PS, although not all PMPs bind to annexin V. The proportion of PMPs that bound annexin V may be dependent on the agonist used to trigger their release, with physiological agonists such as collagen resulting in fewer annexin V+ MPs, than non-physiological agonists, such as calcium ionophore (Connor *et al.*, 2010).

3.4.1.2. Stimulation of Leucocytes to Produce Leucocyte-derived Microparticles *In Vitro*

CD45+ LMPs, CD66B+ granulocyte-derived MPs and CD14+ monocyte-derived MPs were all significantly elevated following stimulation of whole blood with PMA and calcium ionophore. PMA binds to and activates protein kinase C, causing cell differentiation, and calcium ionophore causes cell activation and proliferation. CD45, CD66B and CD14 are well-validated markers for leucocytes, granulocytes and monocytes respectively. This suggests that CD45, CD66B and CD14 markers able to bind to MPs generated *in vitro*, and therefore they will be used as markers for MPs in the plasma of healthy controls and patient groups discussed in this thesis.

Granulocyte-derived MPs have previously been triggered from neutrophils primed with TNF-α and stimulated with ANCAs. These MPs can increase the expression of adhesion molecules on endothelial cells and up-regulate the release of IL-6 and IL-8 from endothelial cells (Hong *et al.*, 2012). Conversley, granulocyte-derived MPs from N-formyl-methionyl-leucyl-phenylalanine (fMLP) stimulated neutrophils, were found to be rich in the anti-inflammatory protein annexin 1, and they were able to inhibit neutrophil

adhesion to endothelial cells (Dalli *et al.*, 2008). Therefore, granulocyte-derived MPs may have either a proinflammatory or an anti-inflammatory role, depending on the stimuli which resulted in their generation.

3.4.1.3. Stimulation of RBCs to produce RBC-derived Microparticles *In Vitro*

In this study, glycophorin A+ RBC-derived MPs were significantly increased following stimulation with PMA and calcium ionophore. Numbers of glycophorin A+ RBCs were significantly decreased following stimulation, suggesting that this stimulation results in the breakdown of RBCs to produce RBC-derived MPs. Glycophorin A is a well defined marker for RBCs, and also successfully labelled MPs in this study. It was previously found that calcium ionophore activated RBCs release MPs, and that these MPs were antigenically and functionally different from their parent cells (Salzer *et al.*, 2002).

3.4.2. Stimulation of HUVECs to Produce Endothelial Cell-Derived Microparticles *In Vitro*

3.4.2.1. TNF-α Stimulation of HUVECs

TNF-α stimulation of HUVECs for 25 hours led to a significant increase in annexin V+ EMPs, lactadherin+ EMPs, CD31+ EMPs, CD62E+ EMPs, CD106+ EMPs and CD144+ EMPs. This is supported by Coombes *et al*, who found an increase in CD31+ and CD62E+ EMPs following 24 hour TNF-α stimulation. They also found that CD62E was not present on resting HUVECs, but that its expression was induced by TNF-α stimulation (Combes *et al.*, 1999). Another study reported that TNF-α stimulation of HUVECs generated EMPs that expressed annexin V+ and CD31 (Philippova *et al.*, 2011).

3.4.2.2. IL-1α Stimulation of HUVECs

IL-1α stimulation for 25 hours led to a significant increase in annexin V+ EMPs, lactadherin+ EMPs, CD31+ EMPs, CD62E+ EMPs and CD144+ EMPs. This is supported by Abid Hussein *et al* (2003), who found that IL-1α stimulation of HUVECs for 12 hours caused a significant increase in CD62E+ EMPs. They also found that CD31 was constitutively expressed on HUVECs, but only a sub-population of EMPs expressed it and that CD106 and CD144 were hardly detectable on EMPs, even following activation, despite being identifiable and up-regulated on HUVECs. This may explain why very few CD106+ EMPs were found, following stimulation with various agonists. A significant increase in the release of CD144+ EMPs was only found following stimulation with IL-1α at 50 ng/ml, suggesting that the release of these EMPs requires greater stimulation. This fits with the concept that CD144+ EMPs are a marker of endothelial cell apoptosis (Jimenez *et al.*, 2003b).

3.4.2.3. IL-6 Stimulation of HUVECs

Data in this chapter showed that 25 hours stimulation with IL-6 caused a significant increase in annexin V+ EMPs, lactadherin+ EMPs, CD31+ EMPs and CD144+ EMPs, compared to media alone, suggesting that IL-6 triggers their release. This is supported by the findings that CD31+ EMPs are positively associated with levels of IL-6 in healthy individuals (Chirinos *et al.*, 2005b). CD31 and CD144 are EMP markers indicative of endothelial cell apoptosis, as opposed to activation, suggesting that IL-6 may result in endothelial cell death.

3.4.2.4. CRP Stimulation of HUVECs

CRP stimulation of HUVECs led to an increase in CD31+ EMP release. Confirming the findings of Wang *et al* (2007), who reported that CRP induced the release of CD31+ from HUVECs. They suggest that CRP levels are able to mediate endothelial cell apoptosis, by reducing nitric oxide (NO) formation, and therefore increasing EMPs produced by apoptosis. This hypothesis is supported by Verma *et al* who showed that CRP can impair endothelial function, by attenuating the bioavailability of NO (Verma *et al.*, 2002). Treatment of human aortic endothelial cells with CRP also results in an increase of EMPs, and this was suggested to be one of the mechanisms by which CRP leads to endothelial dysfunction in CVD (Devaraj *et al.*, 2011)..

3.4.2.5. Summary of Stimulation of HUVECs to Produce Endothelial Microparticles

CD106+ EMP release from HUVECs was only increased by TNF-α stimulation at 100 ng/ml. CD106 is known to be up-regulated on endothelial cells in response to TNF-α and IL-1 (Melendez *et al.*, 2003), and would therefore, be expected to be present on EMPs released from these cells. The findings in this Chapter may suggest that HUVECs do not readily release CD106+ EMPs in response to the concentration of agonists used here.

Moderate levels of annexin V+, lactadherin+ and CD31+ EMPs were produced from HUVECs in the media alone well after 25 hours, as shown in Figures 3.4-3.6. This may be due to the stress of being cultured for 25 hours in the small well. It could also suggest that the un-stimulated endothelial cells release MPs under resting conditions.

The release of EMPs from HUVECs can be triggered by several agonists and the data here support the hypothesis that the antigenic composition of the EMPs released is dependent on the agonist used. Jimenez *et al* (2003) also found that the markers expressed on EMPs were dependent on the stimuli used to trigger them. If this is the case *in vivo* as well, then it would explain why different diseases cause different EMP profiles. For this reason, all of the EMP markers discussed in this Chapter will be used to label EMPs in the plasma of the healthy individuals and patient groups described in subsequent Chapters.

3.4.3. Limitations

It is acknowledged that the stimulation of leucocytes and RBCs was carried out in whole blood, and therefore the cell specificity of the MPs produced could not be identified. Also these experiments were only carried out in whole blood from three individuals. The T-cell MP marker (CD3) was not examined. Ideally granulocytes, monocytes, T-cells and RBCs would have been individually isolated from blood of several individuals, or grown as individual cultures, prior to stimulation, in order to confirm the specificity of the cell-specific markers. However, these stimulation experiments did show that the cell-specific markers were capable of binding MPs produced from stimulated whole blood.

Not all stimulations of cells led to the release of MPs in a dose-dependent manner. For example, CD45+ LMP and CD66B+ granulocyte-derived MP release was increased following stimulation with the first two concentrations of PMA and calcium ionophore, but these levels decreased at the highest concentration. CD106+ EMPs were significantly increased following stimulation with 100 ng/ml, but not 1000 ng/ml TNF-α. Also, 5 ng/ml of IL-1α was more successful at stimulating the release of EMPs than 50 ng/ml. This suggests that the very high concentrations of agonist may be causing cell death by necrosis, instead of leading to MP release during cell activation or apoptosis.

The results shown for the HUVEC stimulation experiments were the mean and SEM of only three staining experiment repeats. Ideally the stimulation of HUVECs would have been carried out multiple times to ensure that the same pattern of EMP release was observed with each agonist.

The *in vitro* generation of MPs from isolated cells or cell cultures gives some information regarding the antigenic composition of MP subtypes. However, *in vitro* generated MPs do not necessarily correspond directly to the MPs released *in vivo*. Therefore, although the cell-specific markers were able to stain *in vitro* generated MPs, it does not necessarily infer that they would stain MPs *in vivo*.

3.4.4. Conclusions

Stimulation of platelets, leucocytes and RBCs led to the generation of MPs from these cell types. These *in vitro* generated MPs bound to the cell specific makers chosen for use in this PhD, supporting the validity of their use.

Stimulation of cultured endothelial cells with various physiological agonists, led to an increased release of EMPs expressing the EMP markers chosen for use in this thesis. The phenotype of the EMPs released appeared to be dependent on the agonist used to stimulate their release.

Chapter 4 – Alternative Assays for the Measurement of Microparticles

4.1. Introduction

4.1.1. Limitations of Flow Cytometry

Although flow cytometry is the most commonly used method for MP analysis, it does have limitations. It cannot be used to detect MPs smaller than a certain diameter. Depending on the threshold of the machine, this has been stated to be between 0.3-0.5 μm (Beyer and Pisetsky, 2010; Nomura *et al.*, 2008; Shantsila *et al.*, 2010). The majority of MPs are now thought to be less than 0.3 μm in size (Freyssinet *et al.*, 2009). Therefore, flow cytometry may just be measuring the 'tip of the iceberg' when analysing MPs. In order to measure the smaller particles it would be necessary to use an alternative technique, such as electron microscopy.

The use of isotype controls in the flow cytometric analysis of MPs may also be a source of variation. It has been found that the diversity in staining intensity of isotype controls, can lead to a large CV% and errors in the quantification of MPs (Trummer *et al.*, 2008). Fluorescence-minus-one (FMO) control gating is a more accurate strategy for MP analysis. However, it is not practical to use FMO staining on each run of MP analysis, as it requires several extra control tubes each time.

MPs do not express the same surface markers at the same level as their cells of origin, making it difficult to identify certain MP subtypes. Also flow cytometry analysis of MPs results in increased background staining, as there is more space between the MP and the sheath tube, for unbound antibodies to be detected.

This PhD project will mainly focus on the use of flow cytometry to detect MPs, because of its ability to detect and quantify MPs from different cellular origins. However, due to the above mentioned limitations, it was of interest to investigate other MP assays. It is recognised that these other assays have logistical advantages over flow cytometry, such as 96-well formats and semi automation options. Also, the alternative assays investigated give functional information about the circulating MPs.

4.1.2. Well Established Microparticle Assays

There are several other well-established methods that have been used to analyse MP populations in PPP, as reviewed by Jy *et al* (2004).

4.1.2.1. Enzyme-linked Immunosorbant Assay (ELISA)

ELISA capture methods involve coating the plate with annexin V or a cell-surface marker of interest. Plasma is added to the plate, then the plate is washed and a cell specific antibody is added, to detect the MP subtype of interest. Commercial assays are available, which have been standardised. ELISAs are ideal for analysing large batches of samples, as they can be easily automated. However, they can not detect MPs from several different cellular origins at the same time and they give no size indication.

4.1.2.2. Procoagulant Activity Assays

Procoagulant activity based assays exploit the functional capability of the MPs. They involve coating a micro-titration plate with annexin V to capture the MPs from the plasma. Prothrombin is then added and the amount of thrombin produced is measured, as this correlates with the number of procoagulant MPs in the plasma. Commercially available chromogenic assays are available, including one to quantify tissue factor activity in MP samples (Yuana *et al.*, 2011). These methods can be easily automated, are relatively low-cost and give functional information about MPs, but they only quantify the procoagulant MP population, and they give no size information.

4.1.2.3. Electron Microscopy (EM)

EM can be used to analyse MPs, and to give information on their size, shape and internal architecture. MPs can be immunogold-labelled with annexin V and cell-specific markers, and then visualised by whole-mount immuno-electron microscopy. This method can also be used to analyse small MPs and exosomes, however, it is not quantitative and requires extensive sample preparation, making it impractical for large-scale routine use. Also EM procedures require a vacuum environment and dehydration of the sample, which may affect the MP morphology (Yuana *et al.*, 2011).

4.1.3. Alternative Microparticle Assays Investigated

To examine whether the results obtained from the quantitative flow cytometry method described in Chapter 2, correlate with results given by functional MP assays, three alternative methods were investigated.

4.1.3.1. Zymuphen MP ELISA

The Zymuphen ELISA (HYPHEN BioMed, Quadratech Diagnostics, UK) is a functional assay which measures the procoagulant activity of MPs in platelet-poor-plasma (PPP). A microtitre plate is pre-coated with annexin V-streptavidin, so it only captures annexin V+ MPs. MPs bind to the plate and expose their phospholipid surface, allowing Factor Xa-Factor Va (pro-thrombinase complex) to cleave prothrombin into thrombin. The phospholipid concentration in the sample is the limiting factor, so there is a direct relationship between the phospholipid concentration on MPs, and the amount of thrombin generation. Thrombin generation is measured by a specific chromogenic substrate, and this gives an indication of the annexin V+ MP level in the sample (Owens and Mackman, 2011).

4.1.3.2. STA® Phospholipid (PPL) Procoag Assay

STA® PPL Procoag assay (Stago, Berkshire, UK) measures the procoagulant activity of MPs by determining the clotting time for a sample (Owens and Mackman, 2011). The clotting time is dependent on the procoagulant phospholipids of the sample. A shortened clotting time indicates an increase in procoagulant phospholipids (van Dreden *et al.*, 2009), which is proportional to the procoagulant MP levels in the sample.

4.1.3.3. Endogenous Thrombin Potential (ETP) Assay

Thrombin generation can be assessed using a calibrated automated thrombogram. The ETP assay (Stago, Berkshire, UK) measures thrombin generation in a PPP sample. The platelet-rich-plasma (PRP) reagent (tissue factor and a minimal amount of phospholipids) is added initially and acts as a trigger to initiate thrombin generation. At fixed time intervals, a sample is taken from the reaction and the amount of thrombin generated is plotted to give a thrombin generation curve. From this curve, several read-outs can be determined including the lag-time, the time to peak, the peak of thrombin generation, and the area under the thrombin generation curve (ETP). When using PPP samples, the thrombin generation is attributable to the procoagulant MP concentration of that sample.

4.1.4. Aims of this Chapter

1) To investigate alternative well-established assays for the measurement of procoagulant MPs and to determine if results correlate with each other

2) To determine if the results from these functional assays correlate with the flow cytometry quantitative results.

4.2. Methods

4.2.1 Sample Selection

26 healthy control samples were obtained from laboratory staff. Subjects were eligible if aged 18 or above and in good health. Subjects with a known cardiovascular condition or a known cardiovascular-associated condition were excluded. This study was approved by the Oxford Research Ethics Committee (REC No: 11/SC/0183). 27 healthy control samples were obtained from a study investigating sleep and psychomotor performance at altitude. Subjects were eligible if they were healthy males, between 18 and 70 years old. Subjects with a body mass index (BMI) <18 or >30 kg/m2, or any medical condition requiring treatment were excluded. This study was approved by the Zurich ethics committee (KEK ZH 2010-0054/1) and registered (NCT01130948).

47 obstructive sleep apnoea (OSA) patient samples were used to examine samples with elevated numbers of MPs. Patients were eligible if they were aged between 45 and 75 years, had proven obstructive sleep apnoea. The study was approved by the Oxford Research Ethics Committee (REC No: 05/Q1604/159). Written informed consent was obtained from all participants.

In total, 100 samples from healthy individuals (n=53) and OSA patients (n=47) were used to give a wide range of MP levels.

4.2.2. Flow Cytometry Analysis of Microparticles

Samples were analysed by the flow cytometric assay described in Chapter 2. All cell-specific markers described in Chapter 2, were used to stain for MPs.

4.2.3. Alternative Assays Investigated

4.2.3.1. Zymuphen MP ELISA

The PPP samples and kit controls were diluted 1:20 in the kit sample diluent, which is supplemented with calcium, Factor Xa (FXa) and thrombin. A calibrator curve was produced by serial dilutions of the kit calibrant. The calibrator curve, diluted controls and diluted samples were added to the ELISA plate and incubated for 1 hour at 37 °C. The plate was washed 5 times in wash solution, then 100 µl of Reagent A (Fxa-FVa) and 50 µl Reagent 2 (prothrombin) were added, and incubated for 10 min at 37 °C. 50µl of Reagent 3 (thrombin substrate) was added, and incubated for 3 min at 37 °C. Finally 50 µl of stop solution was added, and the plate was read at 405 nm, subtracting the blank. Results were calculated from the calibrator curve, and are expressed as nanomolar (nM) PS equivalent.

4.2.3.2. STA® Phospholipid (PPL) Procoag Assay

PPP samples were tested on the STA® automated analyser (Stago, Berkshire, UK), using the STA® procoag-PPL kit, according to the manufacturer instructions. Two kit controls, with a known clotting time, were analysed first to check the reproducibility of the assay. Samples were loaded and detection of procogulant phospholipids was automatically carried out. PPP was automatically diluted with phospholipid-depleted plasma and incubated for 120 seconds at 37 °C. The addition of reagents permits the triggering of the coagulation cascade downstream from Factor Xa. The clot formation is dependent only on the procoagulant phospholipids contained in the plasma sample. A shortened clotting time indicates increased concentration of phospholipids. The final result is expressed as the clotting time in seconds.

4.2.3.3. Endogenous Thrombin Potential (ETP) Assay

The ETP assay was performed according to the manufacturer instructions. 80 µl of PPP sample was added in duplicate to wells in a 96-well plate in duplicate. 20 µl PRP reagent was added to one set of wells. 20 µl thrombin calibrator was added to the other set of wells. The thrombin calibrator corrects for donor-to-donor differences in colour of plasma and inner filter effects (Owen *et al.*, 2011). The plate was warmed to 37 °C, and then placed on the reader, a Fluoroskan Ascent FL (Thermo Electron Corporation, Helsinki, Finland). 20 µl of Fluca-Kit Reagent, which causes the citrated plasma to be re-calcified to start the reaction, was automatically added to each well. The fluorescence intensity was continuously measured by the Fluoroskan and thrombin generation was plotted over one hour. The computer software calculates lag time (min), Peak (nM), ETP (nM x min) and time to Peak (min).

4.2.3. Statistics

All statistics were performed using GraphPad Prism 5 Software (GraphPad Software, San Diego). The results from each alternative MP assay were compared to the results from the other assays and correlations were determined by Spearman Correlation. A p-value of <0.05 was considered statistically significant.

4.3. Results

4.3.1. Samples Analysed

All 100 selected samples were analysed on the Zymuphen MP ELISA. Due to the required sample volume (>250 µl PPP), only 69 of the 100 samples could be run on the PPL assay. Also due to the sample volume requirement (>160 µl PPP) only 78 of the 100 samples were run on the ETP assay.

4.3.2. Correlations between Alternative Assays

In order to establish if the three alternative assays were giving similar results, correlation analysis was carried out. Table 4.1 and Figure 4.1 show the Spearman r-values and the p-values for correlations between the results from the alternative assays.

Table 4.1 - Associations between Alternative Assays

	Zymuphen ELISA		PPL Procoag Assay		ETP Assay – ETP		ETP Assay - Peak	
	Spearman r	P-value	Spearman r	P-value	Spearman r	P-value	Spearman r	P-value
Zymuphen MP ELISA			-0.5308	<0.0001	0.5498	<0.0001	0.5638	<0.0001
PPL Procoag Assay	-0.5308	<0.0001			-0.7672	<0.0001	-0.7244	<0.0001
ETP Assay – ETP	0.5498	<0.0001	-0.7672	<0.0001			0.9852	<0.0001
ETP Assay – Peak	0.5638	<0.0001	-0.7244	<0.0001	0.9852	<0.0001		

Analysed by Spearman correlation

The ETP assay gives several readouts for the results, including the ETP (nM x min) and the Peak (nM). These two results were very closely correlated ($r = 0.9852$, $p<0.0001$).

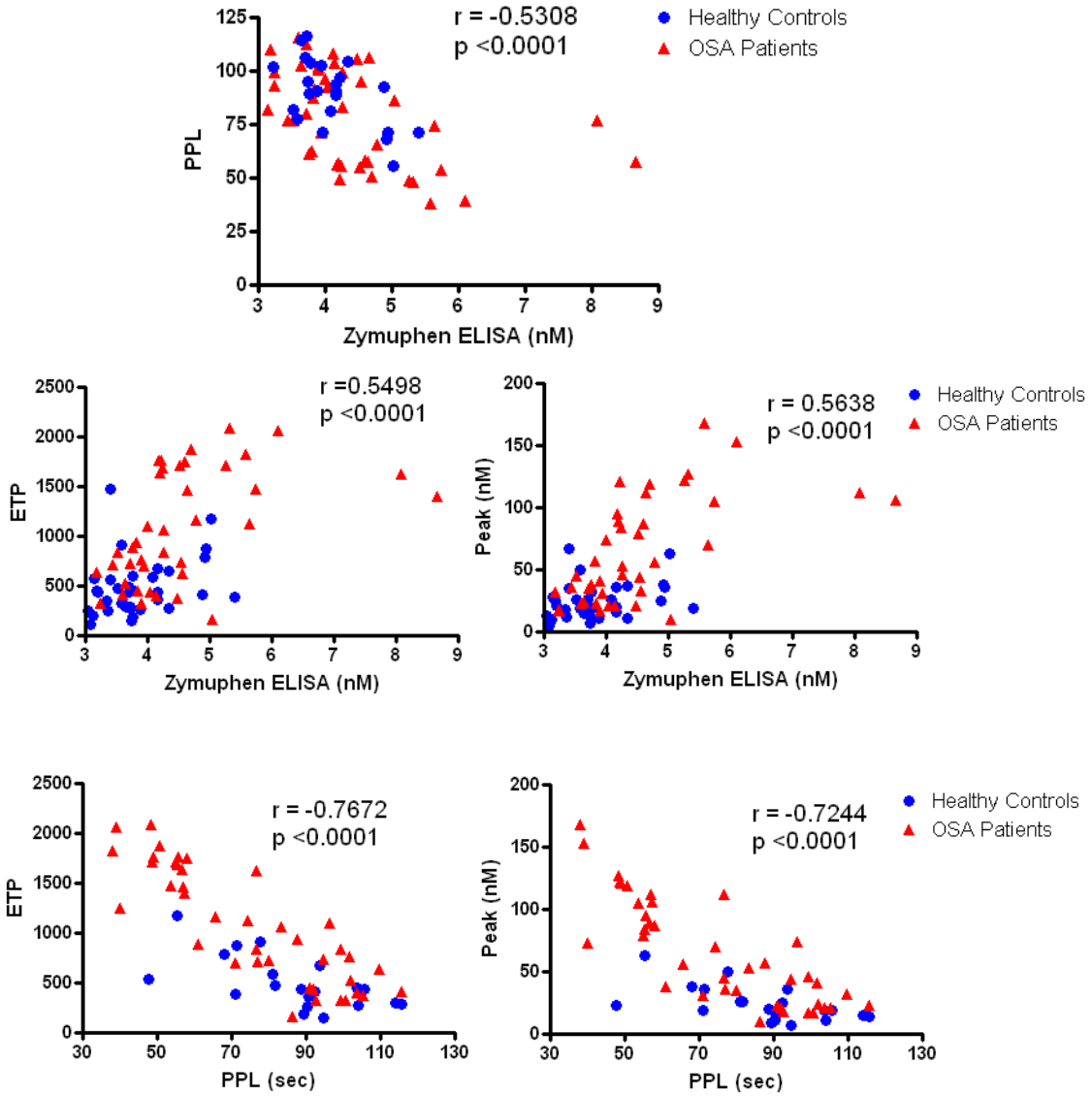

Figure 4.1. Associations between results from the alternative assays. Analysed by Spearman correlation. Zymuphen (nM), PPL (seconds), ETP (nM x min), Peak (nM).

The results from the Zymuphen ELISA are weakly negatively associated with the STA® PPL Procoag assay, and weakly positively associated with the ETP and the Peak results from the ETP assay.

The results of the STA® PPL Procoag assay are negatively associated with the ETP and the Peak from the ETP assay, and are weakly negatively associated with the Zymuphen ELISA.

The ETP and Peak results from the ETP assay are weakly positively associated with Zymuphen ELISA and negatively associated with STA® PPL Procoag assay.

4.3.3. Correlations Between Alternative Assays and Flow Cytometry

In order to establish if the functional MP results from the alternative assays were associated with the quantitative results from the flow cytometry assay, correlation analysis was carried out. Table 4.2 and Figure 4.2 show the Spearman r-values and the p-values for correlations between the results from the alternative assays and the results determined by flow cytometry.

Table 4.2 - Associations between Alternative Assays and Flow Cytometry Results,

	Zymuphen ELISA		PPL Procoag Assay		ETP Assay - ETP		ETP Assay - Peak	
Flow Cytometry Levels	Spearman r	P-value	Spearman r	P-value	Spearman r	P-value	Spearman r	P-value
Annexin V+ MPs	0.5207	<0.0001	-0.7297	<0.0001	0.6427	<0.0001	0.6550	<0.0001
Lactadherin+ MPs	0.5370	<0.0001	-0.7872	<0.0001	0.7444	<0.0001	0.7505	<0.0001
CD31+CD41+ PMPs	0.5203	<0.0001	-0.8632	<0.0001	0.7268	<0.0001	0.7084	<0.0001

Analysed by Spearman correlation

The results from the Zymuphen ELISA are weakly positively associated with annexin V+ MPs, lactadherin+ MPs and PMPs as determined by flow cytometry.

The results of the STA® PPL Procoag assay are negatively associated with annexin V+ MPs, lactadherin+ MPs and PMPs as measured by flow cytometry.

The ETP and Peak results from the ETP assay were positively associated with annexin V+ MPs, lactadherin+ MPs and PMPs results from the flow cytometry assay.

None of the alternative assays were associated with any other MP subtype, such as EMPs, LMPs or RBC-derived MPs, as measured by flow cytometry (data not shown).

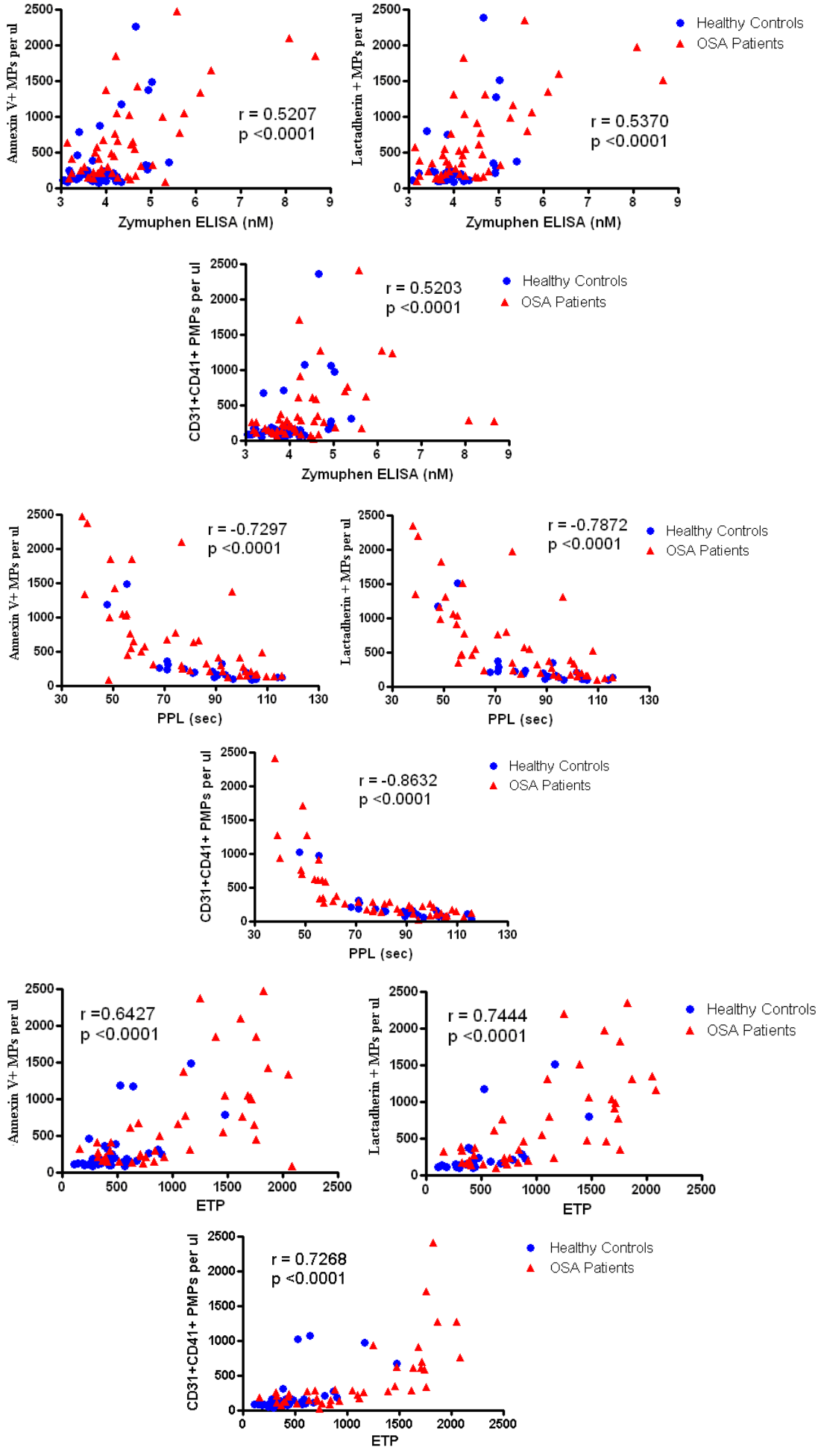

Figure 4.2 - Associations between alternative assays and flow cytometry, analysed by Spearman correlation.

4.4. Discussion

This chapter compared the quantitative results from the flow cytometric assay with results from well-established functional MP assays. In general, results from alternative assays correlated well with each other. Procoagulant MPs and PMPs measured by flow cytometry, correlated well with two of these assays. No other MP subtypes were associated with the results from the alternative assays.

4.4.1. Alternative Assays Investigated

4.4.1.1. Zymuphen MP ELISA

The Zymuphen MP ELISA can be semi-automated and is suitable for high through-put measurement of procoagulant MPs. However, samples require dilution prior to use in this assay, which increases the assay time and chance of human error.

The results from this assay were weakly positively associated with ETP and Peak from the ETP assay, and weakly negatively associated with the Stago PPL assay. It was expected that this assay would correlate well with annexin V+ MPs, as it directly binds annexin V+ MPs. However, it was only weakly positively associated with procoagulant MPs, and this association was not as strong as the other two assays. These findings are supported by Pagana and Chandler (2011), who noted a weak correlation between PMPs measured by flow cytometry and the results from the Zymuphen MP ELISA. This poor association could be due to the Zymuphen MP ELISA measuring smaller MPs, which can not be detected by flow cytometry.

4.4.1.2. STA® Phospholipid (PPL) Procoag Assay

The STA® PPL Procoag assay is fully automated and makes use of an analyser currently used in routine haematology laboratories, so it benefits from being easy to integrate into a routine laboratory. However, it does require that a normal range is established in each laboratory, using at least 20 normal plasma samples.

The STA® PPL Procoag assay was negatively associated with ETP and Peak results from the ETP assay, and weakly negatively associated with the Zymuphen ELISA results. It would be expected to be negatively correlated with the other assays, as the shorter the PPL clotting time, the more procoagulant phospholipids available in the sample. This assay was also negatively associated with annexin V+ MPs, lactadherin+ MPs and PMPs, suggesting that it would be a suitable alternative for measuring procoagulant MPs. This is supported by the data from two other reported studies who also found strong negative

correlations between PMPs detected by flow cytometry and the results from the STA® PPL Procoag assay (Pagano and Chandler, 2011; Stagnara *et al.*, 2012).

4.4.1.3. Endogenous Thrombin Potential Assay

ETP is a useful semi-automated assay which gives results both on the thrombin generation and the rate of generation. 48 samples can be run in duplicate on a 96 well plate and it is relatively cheap. This assay can be standardised and it gives a functional measurement of MPs, however it does not give information on the cell of origin or the size of the particle.

The ETP and Peak results were both positively associated with annexin V+ MPs, lactadherin+ MPs and PMPs. Both ETP and Peak appear to be good predictors of procoagulant MPs present in a sample. This is supported by the findings in a study on MPs in ageing, where an association between annexin V+ MPs measured by flow cytometry and the peak measured by thrombin generation was found (Emmerechts *et al.*, 2012). Another study also reported a positive correlation between PMP results from flow cytometry and peak thrombin generation (Stagnara *et al.*, 2012).

4.4.1.4. Summary of Alternative Assays

These alternative assays have been developed to detect procoagulant MPs. As expected they correlate with each other, and with annexin V+ MPs, lactadherin+ MPs and PMPs. No associations were found between these assays and other MP subtypes, such as EMPs, LMPs or RBC-derived MPs. EMPs have previously been found to be mainly annexin V negative (Ahn *et al.*, 2004), and this was further demonstrated in Chapter 2, where a significant proportion of EMPs and LMPs (in some cases >50 %) did not bind to annexin V. Therefore, it was unlikely that they would be sufficiently detected by these alternative assays.

These alternative assays provide a quick and relatively cheap method of screening large numbers of samples for procoagulant MPs. They also provide functional information about the MPs, and they can measure particles too small to be detected by flow cytometry. However, they can not give an absolute count of MPs, and they provide no information about the cellular origin of the MPs.

These assays have been very useful for validating the results obtained from the flow cytometric assay. It appears that a range of assays may be necessary to provide the most comprehensive information about the quantitative levels and functionality of circulating MPs.

4.4.2. New Developments in Microparticle Analysis

As well as the described flow cytometric assay and alternative functional assays, there have been a great number of new technologies developed and investigated for their ability to measure MPs. The advantages, disadvantages and clinical utility of these new technologies is discussed below.

4.4.2.1. New Generation Flow Cytometers

The lower limit of detection for conventional flow cytometers is 0.3-0.5 μm in diameter, depending on the threshold of the machine (Beyer and Pisetsky, 2010; Nomura *et al.*, 2008; Shantsila *et al.*, 2010). However, newer flow cytometers have improved their resolution of small particles, down to 0.1 μm, as measured by beads (Shantsila *et al.*, 2010), by increasing the light collection angle to get better resolution. This improves the light collection on scatter, allowing small particle-dedicated instruments to have greater sensitivity. A recent study of high sensitivity flow cytometry found that it gave improved FSc resolution, lower background noise and it detected 8-20 times more MPs than standard flow cytometry in coronary patient samples. The ratio of small to large MPs also varied according to clinical status of patient, suggesting that the size of MPs may provide additional biological information (Robert *et al.*, 2012). However, these machines are not yet widely available.

4.4.2.2. Dynamic Light Scattering (DLS)

The Brownian motion of a particle depends on its diameter, making it possible to determine a particles size, using the Stokes-Einstein equation. DLS gives an average particle size, offering a rapid way to measure MPs of all sizes. However, this technique cannot resolve mixtures of MPs and exosomes as it is biased towards the presence of small numbers of large particles. DLS has been previously used to determine MP sizes in fresh-frozen plasma (Lawrie *et al.*, 2009).

4.4.2.3. Nanoparticle Tracking Analysis (NTA)

NTA individually measures a particles size by video analysis of its Brownian motion and use of the Stokes-Einstein equation. NTA can measure much smaller particles than flow cytometry (down to 0.05 μm), it can resolve MPs and exosomes as it measures particles individually, and it is rapid. However, it may be unable to discriminate other plasma particles, such as lipids (Yuana *et al.*, 2011). New advances have allowed for the identification of surface markers by conjugating quantum dots (2-10 nm particles) to the antibody of interest, making it possible to distinguish MPs. This has been demonstrated successfully in the measurement of placental-derived MPs by NTA (Dragovic *et al.*, 2011).

4.4.2.4. Atomic Force Microscopy (AFM)

This technique measures the surface topography of a sample on a MICA (a sheet silicate mineral) slide coated with a cell-specific antibody, by detecting the movement of a counter-lever over the surface of a slide. The majority of MPs detected are 100 nm in size and range from 10 nm – 475 nm, which is similar to the results found by EM and DLS. This technique counts 1000 fold more MPs than conventional flow cytometry, and can still detect a signal in samples filtered at 0.22 um. This suggests that AFM is a more sensitive method for detecting smaller MPs than flow cytometry (Yuana *et al.*, 2010). However, this method is labour intensive, and MPs need to be isolated and concentrated prior to use, which may affect the morphology and number of MPs detected (Yuana *et al.*, 2011).

4.4.2.5. Impedance Based Cytometry

This technology uses a two chamber system with an ionic current. As a particle moves through the gap between the two chambers, there is a voltage spike, which is proportional to the size of particle. The calculation of particle size is based on the fact that the electronic volume is proportional to the change in the impedance associated with the displacement of electrolyte in a flow cell (Coulter principle). This technology gives better discrimination between platelets and MPs than flow cytometry. It has been used to measure tumour-derived MPs in cancer patients (Zwicker, 2010).

4.4.2.6. Proteomic Analysis

Mass spectrometry-based proteomic analysis allows for the characterisation of the protein composition of MPs, giving more information about their structure and functions. However, the contamination of MPs with plasma proteins, such as albumin and immunoglobulins, can affect the results, making interpretation difficult (Yuana *et al.*, 2011).

4.4.3. Limitations

Associations between the Zymuphen MP ELISA and the flow cytometry results were examined in 100 samples. Sample volume requirements meant that fewer samples could be analysed by the STA® PPL Procoag assay and the Endogenous Thrombin Potential assay. Ideally all 100 samples would have been measured in all three alternative assays.

4.4.4. Conclusions

The levels of procoagulant MPs and PMPs from the flow cytometry method correlate with the alternative MP assay results, suggesting that the quantitative results given by flow cytometry are associated with the functional capability of the MPs.

The alternative assays have the advantages of being standardised, either semi or fully automated and they can give functional information about MPs. However, they can only provide information on procoagulant MPs, and cannot detect other MP subtypes, which may provide crucial information regarding disease. These alternative assays could be useful in screening large populations for procoagulant MPs, but flow cytometry would still be necessary for providing information on other MP subtypes.

New technologies have been developed which can detect smaller MPs and even exosomes. They all have advantages and disadvantages, however, most are unable to give cell-specific phenotyping or functional information, which the older flow cytometry or functional assay techniques can provide. The technology chosen for MP analysis will depend on the clinical question being asked.

Due to the fact that flow cytometry can give quantitative information on a wide range of MP subtypes, it will be used as the assay of choice for the analysis of MPs in healthly individuals and patient groups in this thesis.

Chapter 5 – Circulating Microparticles in Healthy Individuals

5.1. Introduction

5.1.1. Role of Microparticles in Health

5.1.1.1. Platelet-Derived Microparticles

Circulating PMPs are present in healthy individuals, in fact they constitute the largest proportion of circulating cell-derived MPs (Berckmans *et al.*, 2001; Morel *et al.*, 2011a), suggesting that they play a physiological role. This theory is strengthened by findings in individuals with Scott Syndrome. In this extremely rare disorder there is a defect in the platelet membrane remodelling, a lack of PS exposure and an impaired ability to release PMPs, leading to a severe bleeding disorder, with no other deficiencies of platelet adhesion, aggregation or metabolism (Nieuwland and Sturk, 2007). This suggests that PMPs are an essential component of the coagulation pathway. Another very rare bleeding condition, Castaman Syndrome is attributed to a deficiency of PMP formation, despite normal PS exposure (Castaman *et al.*, 1997). The surface of PMPs has been found to be 50-100 fold more procoagulant than the surface of activated platelets (Sinauridze *et al.*, 2007).

Berckmans *et al* (2001) found an inverse correlation between the number of annexin V+ MPs and the levels of thrombin-antithrombin complex in the plasma of healthy participants. This suggests that MPs in the circulation of healthy individuals have an anti-coagulant function, by inducing low amounts of thrombin, which activates protein C.

PMPs also appear to have other physiological roles. *In vitro* generated PMPs can serve as a transcellular delivery system for the chemokine RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted), which plays a role in recruiting leukocytes to sites of inflammation (Mause *et al.*, 2005).

PMPs are maintained at a relatively constant level in healthy individuals. Other markers of platelet activation in healthy individuals suggest insufficient platelet activation to account for this baseline level. There has been speculation that a proportion of these PMPs are maintained by the continuous release from megakaryocytes (Flaumenhaft, 2006).

5.1.1.2. Endothelial-Derived Microparticles

Although present in the circulation at much lower levels, it appears that other MP subtypes also have physiological roles in healthy individuals. A subset of EMPs express Von Willebrand Factor (vWF), a glycoprotein important in coagulation, allowing them to induce platelet aggregation (Jy *et al.*, 2005). EMPs also seem to act as a waste disposal

mechanism for endothelial cells, allowing them to avoid accumulation of toxic components, such as Caspase 3, and ultimately avoid apoptosis. In fact, the blocking of EMP release *in vitro*, triggers endothelial cell apoptosis (Abid Hussein *et al.*, 2007).

5.1.1.3. Leucocyte-Derived Microparticles

LMPs stimulate the release of IL-6 from endothelial cells *in vitro*, suggesting that they act as agonists of inflammation (Mesri and Altieri, 1999). In the appropriate setting, this role of LMPs could be a physiological one, however, in inflammatory disease, elevated LMPs could possibly exacerbate the problem.

5.1.1.4. Granulocyte-Derived Microparticles

Granulocyte MPs may be important in anti-inflammatory processes, through their secretion of transforming growth factor (TGF-β) (Gasser and Schifferli, 2004), and the fact that they contain functionally active annexin 1 (Dalli *et al.*, 2008), a ligand potentially important in the clearance of apoptotic cells (Arur *et al.*, 2003). Therefore, a low level of circulating granulocyte-derived MPs in healthy individuals may be necessary to dampen down inflammatory responses.

5.1.1.5. Monocyte-Derived Microparticles

Monocyte-derived MPs are important carriers of tissue factor, and can contribute to the development of a thrombus at the site of injury. It may be that tissue factor only becomes active once monocyte-derived MPs bind and fuse to activated platelets (Nomura *et al.*, 2008).

5.1.1.6. T Cell-Derived Microparticles

T cell-derived MPs have been identified in the circulation of healthy individuals at low levels (Kornek *et al.*, 2011). The MPs released from T cells have been shown to induce degranulation and IL-8 release from mast cells, suggesting that they may carry mast cell activating factors, possibly including OX40, a co-stimulatory molecule (Shefler *et al.*, 2010). It may be that in healthy individuals, T cell-derived MPs could convey surface molecules to other cells, which are not in direct contact with T cells.

5.1.1.7. Red Blood Cell-Derived Microparticles

RBC-derived MPs are present in the circulation of healthy individuals (Chaar *et al.*, 2011). The release of MPs from RBCs is an essential part of the erythrocyte ageing process, by removal of damaged membrane molecules, it can prevent the early removal of otherwise functional RBCs (Tissot *et al.*, 2010). RBC-derived MPs may also have anti-inflammatory properties, including down-regulating macrophages. RBC-derived MPs are present in blood stored for transfusion, and they could account for some of the immuno-suppressive properties attributed to blood transfusions (Sadallah *et al.*, 2008).

5.1.2. Reported Normal Ranges of Microparticles

There has been rapidly growing interest in the role of MPs in disease. However, there are few published reports on the levels of MPs in healthy individuals. Studies often report healthy control MP levels in comparison to their patient MP levels, but due to the variability in the selection of healthy controls, low numbers of healthy controls, and the difference in techniques used to measure MPs, the range of levels reported in healthy individuals is extremely variable (Table 5.1).

Table 5.1 – Healthy Control MP Levels

Reference	Centrifugation Protocol (for PPP)	No of Healthy Controls (M/F)	Annexin V+ MPs	PMPs	EMPs	Other MPs
(Berckmans *et al.*, 2001)	1550 g, 20 min	15		**237** (116-565)	CD62E **64** (16-136)	Granulocyte MPs **46** (16-94) RBC MP **28** (13-46)
(Vince *et al.*, 2009)	180 g, 10 min, 13,000 g, 2 min	8 (all males)			CD106+ **1020** ± 263	
(Tesselaar *et al.*, 2007)	1550 g, 20 min	37 (16M/21F)	**1600** (720-9000)	**1500** (700-7100)		Granulocyte MPs **31** (6-490)
(Bucciarelli *et al.*, 2012)	2880 g, 20 min	418 (120M/298F	**1769** (302-7356)	**1519** (242-7032)		
(Amabile *et al.*, 2008)	500 g, 15 min, 10,000 g, 5 min	20 (7M/13F)	**2991** ± 587	**3163** ± 555	CD62E **75** ± 12 CD144+ **199** ± 33 CD31+CD41- **781** ± 132	LMPs **139** ± 22
(Emmerechts *et al.*, 2012)	1900 g, 10 min, 1900 g, 20 min	18 (10M/8F)		**3488** IQR (2309-6142)		RBC MP **281** **IQR** (194-1507)
(Wang *et al.*, 2007a)	160 g, 10 min, 1000 g, 6 min	76 (31M/45F)			CD31+CD42b- **1111** ± 661	
(Chirinos *et al.*, 2005a)	160 g, 10 min, 1000g, 8 min	25		**5395**	CD31+CD42b- **383** (IQR 243-2403) CD62E+ **223** (IQR 84-315)	

MPs were measured by flow cytometry and levels are events per µl. Levels are given as medians (ranges), or means ± SEM. IQR = Inter Quartile Range.

Table 5.1 demonstrates the immense variability of healthy control levels of MPs reported in the literature. This may be in part due to the different protocols used in the MP analysis, with the initial centrifugation to produce PPP playing only a part in this (Chapter 2). Another problem with the reported healthy levels of MPs is that they are often include only small numbers of participants.

There are currently no published large scale studies on MP levels in healthy controls, which give levels on a wide range of MP subtypes. A published normal range of MPs, for a standardised measurement method, would be an important step towards the introduction of routine MP analysis. This study aims to generate a normal control range for procoagulant MPs, PMPs, EMPs, LMPs, monocyte-derived MPs, granulocyte-derived MPs and RBC-derived MPs, measured by the flow cytometry method described in Chapter 2.

5.1.3. Microparticle Correlations with Participant Characteristics

There is limited published data on the impact of age, gender, body mass index (BMI) and other participant characteristics on the levels of MPs in healthy individuals. It may be hypothesised that MP levels would increase with age, as other cardiovascular risk factors increase. However, a recent study found no significant increase in PMP and RBC MP levels between healthy young and healthy elderly participants (Emmerechts *et al.*, 2012). Bucciarelli *et al* (2011) also found no significant impact of age on total MP levels in 418 healthy controls. In addition, this group found that total levels of MPs were not significantly different between males and females, or between individuals with a low, average or high BMI. There is currently no published comprehensive review of the impact of age, gender and other participant characteristics on levels of a wide range of MP subtypes.

5.1.4. Effect of Hypoxia on Microparticles and Other Inflammatory Markers

Hypoxia is a pathological condition where the whole body or an individual tissue is deprived of oxygen. It can occur in a variety of diseases including obstructive sleep apnoea (OSA), and may be a potential link between OSA and cardiovascular disease (Ryan *et al.*, 2005).

Hypoxia is also one of the stimuli known to induce the release of MPs from cells (Bucciarelli *et al.*, 2012). However, this mechanism was established when observing MP release *in vitro*. For example, non-apoptosis inducing hypoxia leads to the release of vesicles from the human lung tumour cell line (Wysoczynski and Ratajczak, 2009).

Few publications have addressed the role of hypoxia *in vivo* on circulating levels of MPs. One study found that in healthy individuals, breathing of hypoxic air for 80 min led to a significant increase in CD106+ EMPs, however they did not measure other MP subtypes, and they only examined the levels in 8 healthy controls (Vince *et al.*, 2009).

Hypoxia can be induced in healthy individuals by ascent to a higher altitude. Altitudes above 2500 m can cause altitude sickness due to the low partial pressure of oxygen and dehydration. Signs of altitude sickness are a new onset headache at altitudes above 2500 m, with nausea, fatigue, dizziness and insomnia (Hackett and Roach, 2001).

In this Chapter, data was obtained from healthy participants in a randomised, controlled trial primarily investigating the effects of acute exposure to moderate altitude on measures of cardiovascular function and lipid metabolism, to determine the effect of hypoxia on *in vivo* levels of circulating MPs, cytokines and CRP in healthy individuals.

5.1.5. Aims of this Chapter

1) To establish a normal range for circulating MPs in healthy individuals using the flow cytometric assay.

2) To investigate any correlations between MP levels and cardiovascular risk factors, such as age, gender and BMI, in healthy individuals.

3) To determine the effect of hypoxia on *in vivo* levels of circulating MPs and other inflammatory markers in healthy individuals.

5.2. Methods

5.2.1. Microparticle Normal Ranges in Healthy Individuals

5.2.1.1. Subjects

For establishing a normal range, 72 healthy individuals were recruited from two studies. Firstly, 48 healthy control samples were obtained from laboratory staff. Subjects were eligible if aged 18 or above and in good health. Subjects with a known cardiovascular condition or a known cardiovascular-associated condition were excluded. This study was approved by the Oxford Research Ethics Committee (REC No: 11/SC/0183).

24 healthy controls were also obtained from the Early Vascular Study. In this study healthy individuals were examined for their cardiovascular risk factors, and are followed-up long-term. Participants were included if they had no history of CVD. This study was approved by the Oxford Research Ethics Committee (REC No: 06/Q1604/118). Written informed consent was obtained from all participants.

5.2.1.2. Samples for Microparticle Analysis

Citrate blood samples were obtained from all participants, with the first tube following venepuncture being discarded. Within 15 min blood samples were centrifuged at 1550 g for 20 min. The plasma was removed, avoiding the buffy coat, and further centrifuged at 13,000 g for 2 min, to produce PPP. The 250 µl of supernatant was immediately frozen at -80 ºC.

5.2.2. Microparticle Correlations with Participant Characteristics

MP levels in all above participants will be assessed for any associations with age and gender. In addition, participants from the Early Vascular Study will also have measurements taken for their body mass index (BMI), waist/hip ratio and blood pressure (BP). Any correlations between these parameters and MP levels will be examined.

5.2.3. Effect of Hypoxia on Microparticles and Other Inflammatory Markers in Healthy Individuals

5.2.3.1. Subjects

The effect of hypoxia in healthy individuals was examined in samples obtained from a study investigating sleep, breathing and psychomotor performance at altitude. Subjects were eligible if they were healthy males, between 18 and 70 years old. Subjects with a BMI <18 or >30 kg/m2, any medical condition requiring treatment, regular use of medications, a history of altitude related illness were excluded. This study was approved

by the Zurich ethics committee (KEK ZH 2010-0054/1) and registered (NCT01130948). Written informed consent was obtained from all participants.

5.2.3.2. Samples for Microparticle Analysis

On study days all participants were asked to abstain from sporting activities and received standard meals with an energy content of 600-800 kcal three times daily. Venepuncture was performed b etween 6-7 am after an overnight fast. Citrate blood (for MPs) and heparin blood (for other inflammatory markers) samples were taken at 490 m altitude in Zurich and at days 1 and 2 in Jakobshorn at 2590 m altitude. Within 15 min, citrate blood samples were centrifuged at 1550 g for 20 min, to produce PPP. 250 µl of PPP was taken, avoiding the buffy coat, and immediately frozen at -80 ºC. An additional centrifugation at 13,000 g was not possible in this study, due to equipment limitations at altitude, however, all samples were processed identically.

5.2.4. Measurement of Microparticles

MPs were measured by the flow cytometry assay as described in the Chapter 2. All cell-specific markers described in Chapter 2, were used to label MPs.

5.2.5. Measurement of Other Inflammatory Markers

Other inflammatory markers were measured in the effect of hypoxia study. High-sensitivity CRP was measured by the Siemens BN II nephelometry method, which measures a range between 0.18 – 1150 mg/l (Siemens, Surrey, UK). The internal quality control (Siemens Apo-Control-Serum CHD) for high-sensitivity measurement of CRP has a set value and results are expected to be within 10 % of this value.

IL-6, TNF-α and IL-10 were measured using commercially available high-sensitivity ELISAs (BMS213HS, BMS223HS and BMS215HS, Bender MedSystems GmbH, Vienna, Austria). Plates were read on a spectro-photometer (Thermo Scientific Multiskan FC with SkanIt software 2.5.1) at 450 nm primary wave length. The lowest detectable limit was 0.03 pg/ml, 0.13 pg/ml and 0.05 pg/ml respectively. The intra and inter-assay CV% were 4.9 % and 6.0 % for IL-6, 8.5 % and 9.8 % for TNF-α, and 6.8 % and 7.5 % for IL-10. Cytokine measurements were carried out in duplicate and the mean value was reported.

5.2.6. Statistics

Statistics were performed using GraphPad Prism 5 (GraphPad Software, San Diego). Normal ranges are reported as medians (2.5 - 97.5 % percentiles) as MP levels are not normally distributed. Associations between MP levels and participant characteristics were assessed by Spearman correlation. Differences in MP levels between males and females were analysed by Mann Whitney tests. The effect of hypoxia over three time points was assessed by the Friedman Test (a non-parametric repeated measures ANOVA), followed by Dunns Multiple Comparison Test. Non-parametric confidence intervals were calculated using Confidence Interval Analysis Software (CIA Version 1.1). A p-value of <0.05 was considered statistically significant.

5.3. Results

5.3.1. Microparticle Normal Ranges in Healthy Individuals

5.3.1.1. Subject Characteristics

72 healthy individuals were recruited to establish the normal range, 31 males and 41 females. The median age was 29, and ranged from 23-72. All participants were in good health, i.e. no acute infections, at the time of sampling.

5.3.1.2. Normal Ranges of Microparticles

Normal ranges were established from the 72 participants for all MP subtypes (Table 5.2).

Table 5.2 – Normal Ranges of Microparticle Subtypes

MP Subtype	Median (MPs/µl)	2.5 % - 97.5 % Percentile Range (MPs/µl)
Annexin V+ MPs	164.5	55.8 – 690.8
Lactadherin+ MPs	184.7	74.3 – 708.2
CD31+CD41+ PMPs	118.1	31.7 – 446.3
CD31+CD41- EMPs	3.2	0.0 – 19.5
CD144+ EMPs	3.6	0.2 – 55.8
CD62E+ EMPs	4.9	0.0 – 93.8
CD106+ EMPs	4.6	0.0 – 95.5
CD45+ LMPs	10.7	2.7 – 50.6
CD66B+ Granulocyte MPs	4.7	0.1 – 82.4
CD14+ Monocyte MPs	4.4	0.1 – 46.8
CD3+ T cell MPs	2.7	0.0 – 15.8
Glycophorin A+ RBC MPs	8.8	1.3 – 138.7

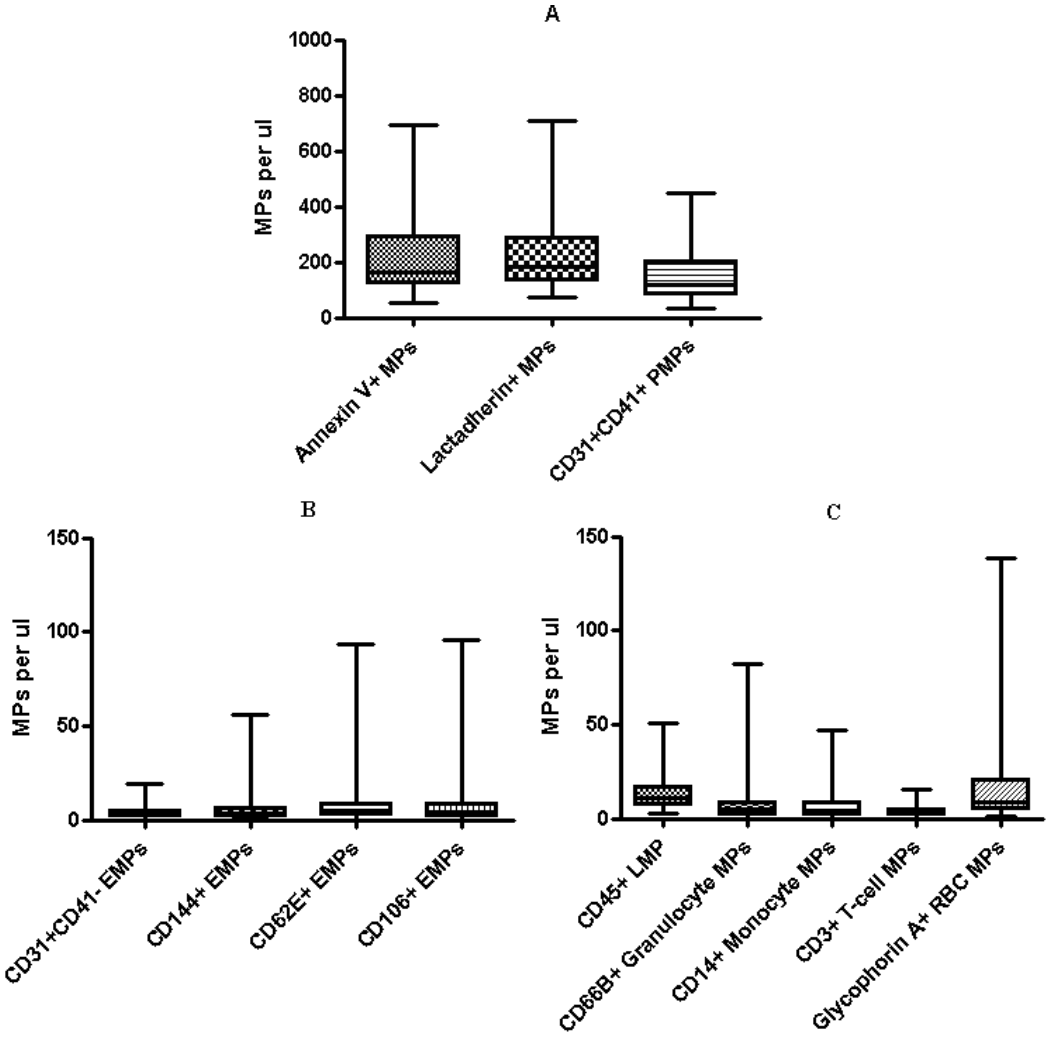

Figure 5.1 – Healthy range of procoagulant MPs and PMPs (A), EMPs (B), LMPs and RBC MPs (C). Whiskers show 2.5 % – 97.5 % percentiles

Procoagulant MPs and PMPs are the most abundant populations in healthy individuals. There is a large range of values for procoagulant MPs, with the majority of healthy individuals having levels between 100 and 300 per µl, but some having levels as high as 700 per µl (Figure 5.1A).

All EMP subtypes are present at much lower levels. Of the EMP subtypes CD62E+ and CD106+ have the largest range of values, with some healthy individuals having levels as high as 90 EMPs per µl (Figure 5.1B).

Other MP subtypes were only detectable at low levels in healthy individuals, in some individuals they were not detectable at all by the flow cytometric assay. RBC-derived glycophorin A+ MPs have the largest range of the other MP subtypes, with some individuals having levels of nearly 140 RBC MPs per µl (Figure 5.1C).

5.3.2. Microparticle Associations with Participant Characteristics

5.3.2.1. Age

No direct correlations were observed between age and any MP subtype.

5.3.2.2. Gender

To identify any gender differences in MP levels, all MP subtypes were compared between healthy males (n = 31) and healthy females (n = 41)

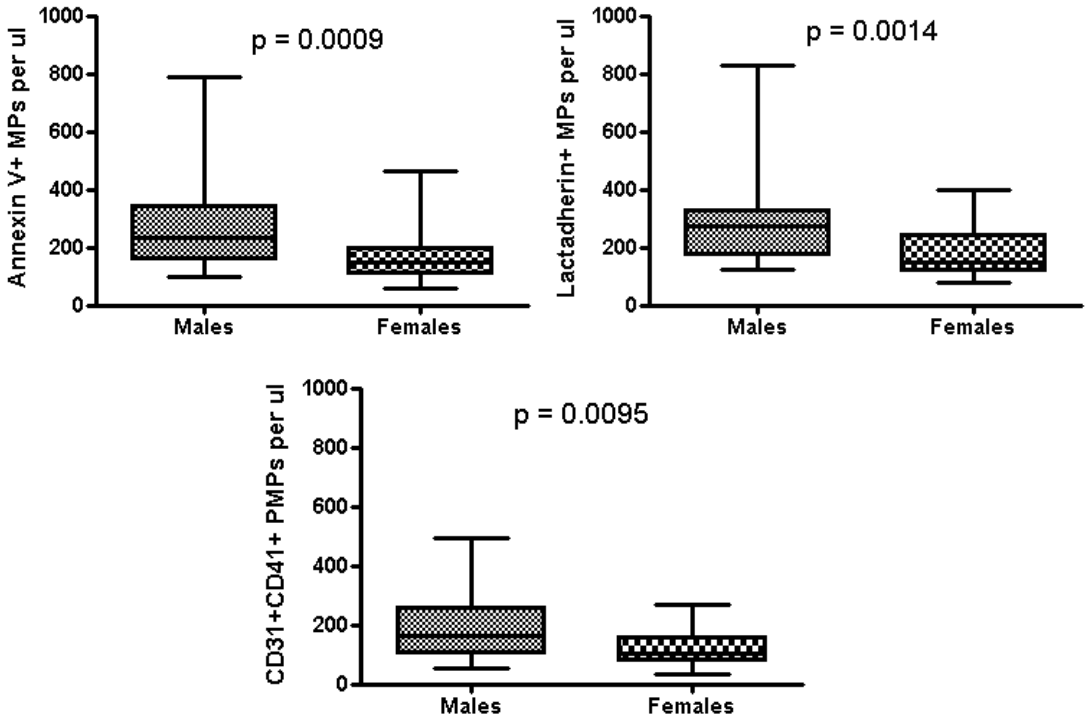

Figure 5.2 – Box and whisker plots (5 – 95 percentile) of procoagulant MPs and PMPs in healthy males and females

Healthy males have significantly elevated levels of procoagulant MPs and PMPs, than healthy females (Figure 5.2). No other MP subtypes were significantly different between males and females (data not shown).

5.3.2.3. Other Factors

24 healthy individuals, recruited through the Early Vascular Study, also had information recorded on their height, weight, BMI, waist/hip ratio, systolic BP and distolic BP. These parameters were assessed with all MP subtypes for any correlations. There were no associations found between these demographics and any MP subtype (data not shown).

5.3.3. Effect of Hypoxia on Microparticles and Other Inflammatory Markers in Healthy Individuals

5.3.3.1. Subject Characteristics

51 male subjects were eligible for entry and completed the study protocol. The mean age of the study population was 26.9 (SD 9.3) years and mean BMI was 23.1 (SD 2.5) kg/m^2.

Blood samples in this study were only centrifuged once at 1550 g for 20 min, prior to freezing. This explains the higher levels of procoagulant MPs and PMPs detected in this study, compared to the other healthy controls.

5.3.3.2. Procoagulant Microparticles

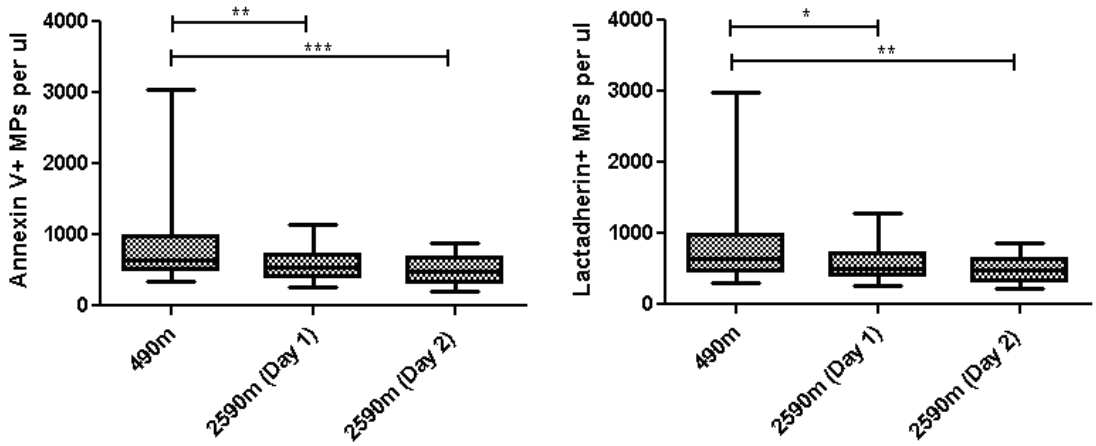

Figure 5.3 – Box and Whiskers plots (5-95 percentile) of annexin V+ and lactadherin+ MPs levels at 490 m and Days 1 and 2 at 2590 m altitiude. MP levels were compared by a Friedman ANOVA, followed by Dunns Multiple Comparison Test (*p<0.05, **p<0.01, ***p<0.001).

Annexin V+ MPs were significantly decreased between 490 m and Day 1 at 2590 m (difference between medians -169/µl, 95% CI -278.3/-79.0, p<0.01) and further reduced on Day 2 at 2590 m (difference between medians -221/µl, 95% CI -370.8/-119.0, p<0.001).

Lactadherin+ MPs were significantly lower on Day 1 at 2590 m (difference between medians -140/µl, 95% CI -258.1/-55.1, p<0.05) and on Day 2 at 2590 m (difference between medians -202/µl, 95% CI -372.2/-93.1, p<0.01) compared to 490 m (Figure 5.3).

5.3.3.3. Platelet-Derived Microparticles

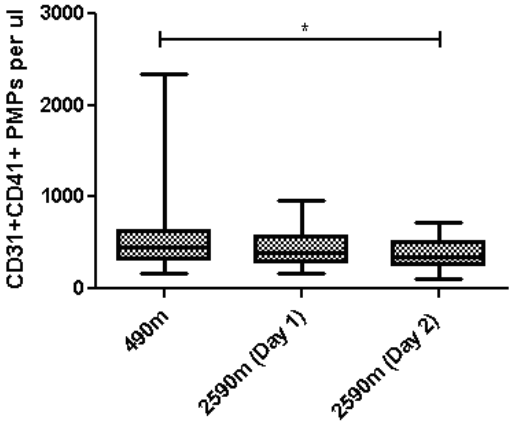

Figure 5.4 – Box and Whiskers plots (5-95 percentile) of PMP levels at 490 m and Days 1 and 2 at 2590 m altitiude. MP levels were compared by a Friedman ANOVA, followed by Dunns Multiple Comparison Test (*p<0.05).

CD31+CD41+ PMPs were not significantly altered between 490 m and Day 1 at 2590 m (difference between medians -56.0/µl, 95% CI -125.9/+4.3, p>0.05). However, they were significantly lower on Day 2 at 2590 m (difference between medians -114/µl, 95% CI -189.9/-51.0, p<0.05) compared to 490 m (Figure 5.4).

5.3.3.4. RBC-Derived Microparticles

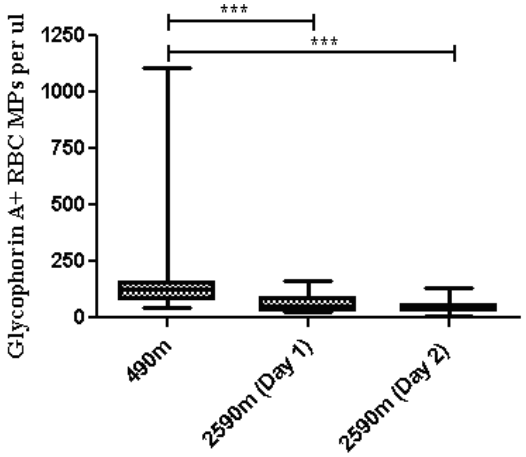

Figure 5.5 – Box and Whiskers plots (5-95 percentile) of Red Blood Cell-derived MP levels at 490 m and Days 1 and 2 at 2590 m altitiude. MP levels were compared by a Friedman ANOVA, followed by Dunns Multiple Comparison Test (***<0.001)

RBC-derived MPs were significantly lower on Day 1 at 2590 m (difference between medians -61.3/µl, 95% CI -87.5/-38.9, p<0.001) and further reduced on Day 2 at 2590 m (difference between medians -81.4/µl, 95% CI -109.9/-57.7, p<0.001) compared to 490 m (Figure 5.5).

5.3.3.5. Other Microparticle Subtypes

Levels of EMPs, LMPs, granulocyte-derived MPs, monocyte-derived MPs and T cell-derived MPs, were not significantly changed by moderate altitude (data not shown).

5.3.3.6. Other Inflammatory Markers

Alongside the measurement of MPs in this study, the levels of other inflammatory markers were also determined.

Table 5.3 – Levels of other Inflammatory Markers at Varying Altitudes

Inflammatory Marker	490 m Attitude (n = 51)	2590 m Altitude Day 1 (n= 51)	2590 m Altitude Day 2 (n = 51)	P-value
CRP (mg/l)	0.44 (0.18 – 0.91)	0.26 (0.17 - 0.53) *	0.33 (0.17 - 0.49) *	P < 0.05
IL-6 (pg/ml)	0.54 (0.28 – 0.94)	0.40 (0.26 - 0.62)	0.43 (0.28 - 0.63)	p > 0.05
TNF-α (pg/ml)	0.77 (0.67 – 0.97)	0.83 (0.62 - 0.98)	0.77 (0.63 - 1.04)	p > 0.05
IL-10 (pg/ml)	0.51 (0.36 – 1.18)	0.56 (0.31 - 1.05)	0.64 (0.34 - 0.92)	p > 0.05

Values are median (quartiles) * $p<0.05$

CRP levels were significantly reduced on both day 1 and day 2 at 2590 m altitude, compared to 490 m altitude. Levels of IL-6, TNF-α and IL-10 were not significantly altered at the higher altitude.

5.4. Discussion

5.4.1. Microparticle Normal Ranges in Healthy Individuals

Levels of MPs in healthy individuals were not normally distributed, and can vary widely. The large spread of MP levels in healthy controls may make it difficult to identify small elevations in MPs in disease states.

Procoagulant MPs and PMPs were the most abundant populations in the circulation of healthy individuals, as shown previously (Chaar *et al.*, 2011; Tushuizen *et al.*, 2006). EMPs and other MP subtypes were present at much lower levels, however, it was possible to consistently detect them using our method, even at very low levels, in the majority of healthy participants.

The levels of EMPs detected were dependent on the surface marker used to identify them. CD62E+ EMPs and CD106+ EMPs have the largest range of values, possibly reflecting the fact that they are generated by endothelial cell activation, which may be occurring in healthy individuals. CD31+CD41- EMPs and CD144+ EMPs are thought to reflect structural damage of endothelial cells (Amabile *et al.*, 2008), and would be expected to be present at much lower levels in individuals who are healthy.

LMPs, granulocyte-derived MPs and monocyte-derived MPs can all be detected in healthy individuals. T cell-derived MPs are present in some healthy people, but at much lower levels and are often undetectable. RBC-derived glycophorin A+ MPs have the largest range of levels of the other MP subtypes, which may be due to differences in erythrocyte aging and turnover between individuals.

The healthy MP ranges found in this Chapter are most similar to those reported by Berckmans *et al* (2001), who use a similar flow cytometric assay. However, they used CD61 as their platelet marker and they centrifuged the plasma once prior to freezing.

5.4.2. Microparticle Correlations with Participant Characteristics

This study showed no significant association between age and any MP subtype. This is supported by a study which found no significant differences in PMP and RBC MP levels in healthy young (median age = 29) and healthy elderly patients (median age = 85) (Emmerechts *et al.*, 2012).

Data in this Chapter indicated that healthy males had elevated levels of procogulant MPs and PMPs compared to healthy females. Ueba *et al* (2010) also found significantly elevated levels of PMPs in healthy males, than healthy females in a large cross-sectional study. As gender differences have been identified in MPs levels in healthy individuals, it is

important to select a healthy control group that is gender-matched to the patient group of interest.

There were no associations between BMI and any MP subgroup found, which is in agreement with Bucciarelli *et al* (2011). There were also no correlations found between hip/waist ratio and BP, with any MP subgroup. These correlations were only assessed in 24 of the healthy controls, where additional data was available. Associations may have been identified if more participants had been examined.

5.4.3. Effect of Hypoxia on Microparticles and Other Inflammatory Markers in Healthy Individuals

In this study, there was a significant reduction in procoagulant MPs, PMPs and RBC MPs, but no significant change in EMPs, LMPs, granulocyte-derived MPs, monocyte-derived MPs and T cell-derived MPs, following hypoxia induced by moderate altitude, in healthy individuals. The reduction in these MP subtypes may be due to a decreased production or an increased clearance or a combination of both.

Procoagulant MPs and PMPs are essential components of the coagulation system, as demonstrated by the severe bleeding disorder Scott Syndrome (Nieuwland and Sturk, 2007). Reports on the effect of hypoxia on coagulation are inconsistent, with some suggesting that hypoxia activates coagulation (Bendz *et al.*, 2000) and others claiming that it has no effect (Crosby *et al.*, 2003). A decrease in platelet count has been noted in healthy individuals exposed to acute hypoxia (Mantysaari *et al.*, 2011). As PMPs are positively associated with platelet counts (Lok *et al.*, 2008), this could account for the reduction in PMPs found here. The findings in this study indicate that MPs, an important coagulation component, are reduced during hypoxia.

Acute hypoxia in an elderly population has been shown to lead to an insufficient antioxidant defence response in erythrocytes and increased erythrocyte lipid damage (de Gonzalo-Calvo *et al.*, 2010). The drop in RBC-derived MPs during hypoxia may reflect an inability to cope with oxidative stress, and may lead to premature deletion of erythrocytes from the circulation.

Data presented by Hefti *et al* as an abstract at the Joint Annual Meeting of the Swiss Society of Pneumology, Peadiatric Pneumology, Thoracic Surgery and Underwater and Hyperbaric Medical Society, supports the findings here. They found a trend for annexin V+ MPs and PMPs to decrease following exposure to high altitude (6210 m) in 8 individuals (Hefti *et al.*, 2012).

Conversely, Vince *et al* (2009) reported a significant increase in CD106+ EMPs in healthy controls following breathing of hypoxic air (15% oxygen, equivalent to 3000 m

altitude) in a temperature controlled laboratory, whereas in this study there was no significant difference in EMPs, even after 48 hours at moderate altitude. However, they measured CD106+ EMPs in only 8 healthy participants, and exposed them to hypoxic conditions for only 80 min, which could explain the confounding results.

Other measurements of cardiovascular function and glucose and lipid metabolism were carried out during this study, and these findings have been submitted for publication in Chest. It was found that at higher altitude, healthy individuals have increased blood pressure and heart rate. However, exposure to altitude actually improved the lipid profile in these individuals, as demonstrated by a significantly increased high density lipoprotein (HDL) and a significantly decreased total cholesterol/HDL ratio and triglyceride levels at 2590 m altitude.

In this study, CRP levels were found to be reduced at higher altitude, suggesting that the acute phase response is dampened down during times of hypoxia. This may explain to some extent the reduced levels of MPs, as levels of CRP and PMPs have previously been shown to be associated (Ueba *et al.*, 2010a).

No significant changes were found in IL-6, TNF-α and IL-10 levels following hypoxia exposure, in this study. This is supported by a study which found that daily exposure to hypoxia in young healthy males, resulted in no significant change in levels of IL-6 (Querido *et al.*, 2011).

Elevated levels of MPs have been identified in patients with OSA (Ayers *et al.*, 2009; Yun *et al.*, 2010) and one hypothesis is that the intermittent hypoxia, caused by the disease, results in increased MP release *in vivo.* The findings here, suggest that hypoxia itself is not a trigger for *in vivo* release of MPs, and that other causes, such as endothelial dysfunction and systemic inflammation, may be responsible for the increased MPs in OSA.

5.4.4. Limitations

Healthy individuals were only included if they had no known cardiovascular conditions or cardiovascular associated conditions. However, some participants may have had underlying conditions, which led to elevated MP levels in some cases. Ethical approval did not permit for a full cardiovascular assessment of all healthy controls.

There was a predominance of females over males in the healthy control group, and it was shown that females have slightly lower levels of procoagulant MPs and PMPs, so this may have skewed the normal range downwards. There was also a predominance of healthy individuals in their 20s, due to inclusion of individuals from the Early Vascular Study.

In order to establish a stable reference range for non-parametric data, it is thought that at least 100 healthy participants are required (Linnet, 2000). However, in this study it was

only possible to recruit 72 individuals. Ideally, more than 100 participants with an even proportion of males and females would have been recruited. With more healthy participants it would also have been possible to report a separate normal range for males and females.

All participants in the effect of hypoxia study were male, so caution should be taken when applying these findings to all healthy individuals. Samples for the hypoxia study were only centrifuged once prior to freezing. However, all samples were processed immediately following venepuncture and identically.

5.4.5. Conclusions

Even in healthy individuals there is a large variation in the levels of MPs detected, making it difficult to identify subtle changes in MP levels between disease and health. However, this work has provided a normal range for a wide range of MP subtypes, using a standardised method, with 72 healthy controls. Healthy males had slightly elevated procoagulant MPs and PMPs, compared to healthy females. All other MP subtypes were similar between males and females. Levels of MPs were not found to be associated with age, BMI or BP.

Hypoxic conditions in healthy individuals, brought about by exposure to moderate altitude, induced a reduction in procoagulant MPs, PMPs and RBC-derived MPs, which is in keeping with the improved lipid profile and reduced CRP levels also found.

Chapter 6 – Microparticles and Other Inflammatory Markers in Obstructive Sleep Apnoea

6.1. Introduction

Obstructive sleep apnoea (OSA) is a sleep disorder characterised by upper airway obstruction during sleep, causing reduced (hypopnoea) or absent (apnoea) ventilation. The airflow is reduced to such an extent that blood oxygen levels fall and a neurological mechanism triggers the individual to wake up (Arias and Sanchez, 2007), resulting in a fragmented sleep pattern. It is characterised by repetitive apnoeas/hypopnoeas during sleep, associated with oxygen desaturations and arousals from sleep.

6.1.1. Epidemiology of Obstructive Sleep Apnoea

OSA is common in western countries, and is estimated to occur in 2 % of middle-aged women and 4 % of middle-aged men (Young *et al.*, 1993). Risk factors for the disease include a high BMI, upper body obesity, male gender and increasing age (Martins *et al.*, 2007). OSA is the third most common serious respiratory disorder after asthma and chronic obstructive pulmonary disease (Chapman *et al.*, 2005). The prevalence of OSA in western countries parallels the progressive increase in obesity (Parati *et al.*, 2007).

6.1.2. Symptoms, Diagnosis and Treatment of Obstructive Sleep Apnoea

Symptoms include loud snoring, with periods of silence, followed by a gasp and resumption of snoring. Therefore, it is often the partner of a sleep apnoea patient who first recognises the disease. Other symptoms include daytime sleepiness, headaches, poor concentration, depression and increased incidence of work and traffic accidents (Patil *et al.*, 2007).

The severity of sleep apnoea is determined by the apnoea-hypopnoea index (AHI) and the oxygen-desaturation index (ODI), measured per hour during sleep. In adults, an apnoea is defined as a cessation, and a hypopnoea as a >30 % reduction, of airflow for at least 10 seconds (Patil *et al.*, 2007). This is determined during an overnight sleep study, where a full polysomnography is carried out (Chapman *et al.*, 2005).

Treatment depends on the severity of the syndrome. Often weight-loss, avoiding alcohol and changing sleeping position can reduce symptoms in patients with minimally symptomatic OSA (Parati *et al.*, 2007). For moderate to severe OSA, treatment with continuous positive airway pressure (CPAP) is necessary. CPAP maintains a generally

constant pressure in the airway, by generating airflows into the upper airway through a nasal or full-face mask (Basner, 2007). The constant airway pressure prevents collapse of the pharynx, thereby stopping apnoeas/hypopnoeas and preventing fragmentation of sleep. CPAP has been shown to improve daytime symptoms, quality of sleep and blood pressure in individuals with OSA (Basner, 2007).

In moderate to severe symptomatic OSA, it has been shown that endothelial dysfunction improves following treatment with CPAP (Cross *et al.*, 2008; Ip *et al.*, 2004). Vascular function is known to be impaired in minimally symptomatic OSA patients when compared to matched control subjects without OSA (Kohler *et al.*, 2008), so these patients may also benefit from CPAP treatment.

6.1.3. Obstructive Sleep Apnoea and Cardiovascular Disease

OSA patients are at a higher risk of developing cardiovascular disease (CVD), including hypertension, cardiac arrhythmias and myocardial infarction. OSA patients have a 4.6 fold increased risk for coronary artery disease (CAD), when compared to a healthy population (Peker *et al.*, 2006). Cross-sectional and prospective studies have implicated OSA as an important causal factor in CVD (Marin *et al.*, 2005; Peker *et al.*, 2002; Peppard *et al.*, 2000; Redline *et al.*, 2010)

Many of the risk factors for OSA, such as increased age, male gender and high BMI, are also risk factors for CVD, making it difficult to directly link the two diseases. However, a study that adjusted data for these risk factors, showed OSA to be an independent risk factor for hypertension and its complications (Lavie *et al.*, 2000). The link between OSA and CVD is not fully understood.

Both the acute and chronic effects of OSA have been implicated in the development of CVD. The acute effects include intermittent hypoxia and arousal from sleep, which lead to fluctuations in heart rate, sympathetic activation and rises in BP. The chronic effects include increased BP, increased oxidative stress, increased coagulation and development of insulin resistance (Arias and Sanchez, 2007), all of which are risk factors for CVD. OSA is also associated with elevations in CRP and proinflammatory cytokines, creating a proinflammatory environment (Minoguchi *et al.*, 2005; Shamsuzzaman *et al.*, 2002).

Endothelial dysfunction is reported in several diseases, including CAD (Neunteufl *et al.*, 1997) and hypertension (Contreras *et al.*, 2000), and it may provide another link between OSA and CVD (Figure 6.1). Studies have found impaired endothelial function in patients with OSA (de la Pena *et al.*, 2008). This may be caused by repetitive hypoxia, changes in shear stress, reduced endothelial repair capacity and vascular reactivity (Lurie, 2011). Treatment with CPAP has been shown to improve endothelial function, by endothelial

nitric oxide (NO) release and stimulating endothelium dependent vasorelaxation in the systemic circulation (Lattimore *et al.*, 2006).

The assessment of endothelial function in OSA has been carried out using a variety of methods, including functional analysis of vascular responses, plasma analysis of inflammatory mediators, brachial artery blood flow, and circulating apoptotic endothelial cells (Budhiraja *et al.*, 2007). MPs could also serve as useful markers of endothelial dysfunction in OSA. LMPs carry the necessary components to cause endothelial dysfunction and EMPs may be generated in the response to endothelial dysfunction.

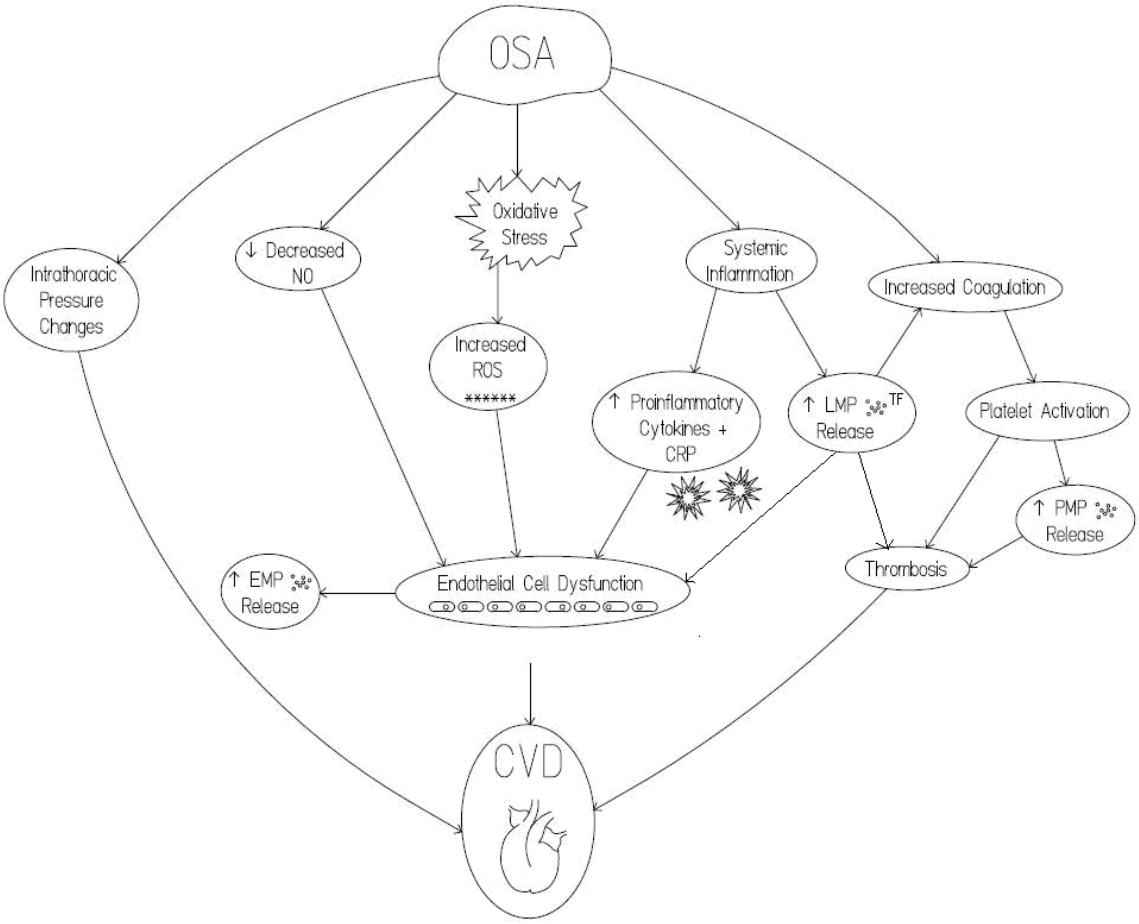

Figure 6.1 – Possible mechanisms linking obstructive sleep apnoea and cardiovascular disease, including MPs

6.1.4. Microparticles and Obstructive Sleep Apnoea

The MSc project preceding this PhD led to a publication in the European Respiratory Journal on the levels of circulating MPs in patients with minimally symptomatic OSA (Ayers *et al.*, 2009). Prior to this, there has been only limited work on MPs in OSA patients. One study found significantly more CD63+ activated platelets in OSA patients compared to healthy controls, but no difference in PMP levels. However, that study only examined 12 OSA patients and 6 healthy controls (Geiser *et al.*, 2002).

My initial study (Ayers *et al.*, 2009) examined annexin V+ MPs, PMPs, EMPs and LMPs in 56 OSA patients and 15 age, gender, BMI and cardiovascular-comorbidity matched controls. Annexin V+ MPs, PMPs and LMPs were all significantly elevated in OSA patients compared to controls and EMPs showed a non-significant tendency to be higher. Annexin V+ MPs and PMPs in OSA patients were twice those found in controls, suggesting that OSA could play an independent role in the formation of MPs. Although controls were carefully matched to the patient group, other unmeasured factors may have accounted for these differences. Therefore, controlled interventional studies were needed to confirm the findings.

Since this publication there has been a growing interest in MPs in OSA. Maruyama *et al* (2012) confirmed our findings of elevated PMPs in OSA patients. Jelic *et al* (2009) found that CD31+CD42- EMPs were significantly higher in OSA patients than controls. These EMPs were inversely correlated with endothelial progenitor cells, a marker of endothelial repair. They also found that four weeks of CPAP treatment, caused a tendency of CD31+CD42- EMPs to decrease. Similarly Yun *et al* (2010) reported that CD31+CD42- EMP and CD62E+ EMP levels were significantly higher in OSA patients than controls. They also found that 4-6 weeks CPAP treatment reduces the levels of CD62E+ EMPs. Another study found that the levels of CD31+CD42- EMPs, CD62E+ EMPs, LMPs and PMPs varied according to the severity of paediatric OSA, and that LMPs and PMPs were significantly associated with AHI (Kim *et al.*, 2011). These studies suggest that MP levels may assist in determining the severity of disease in OSA. However, further work is needed to determine if MP levels are a useful indicator of treatment success.

Few studies have analysed levels of MPs in OSA, and even fewer assess the impact of treatment. Although there is preliminary data from several case-control studies that MP levels are increased in patients with OSA, and that CPAP therapy lowers these levels (Ayers *et al.*, 2009; Jelic *et al.*, 2009; Kim *et al.*, 2011; Maruyama *et al.*, 2012b; Yun *et al.*, 2010), there are no data from randomised controlled trials proving a causal relationship between OSA and increased levels of MPs. This uncertainty was addressed in this Chapter by measuring MP levels in two double-blind randomised controlled studies. The first

determines the effect of six months CPAP treatment in a group of minimally symptomatic patients. The second investigates the effect of a two week CPAP therapy withdrawal on levels of MPs in moderate/severe OSA patients.

6.1.5. Other Inflammatory Markers and Obstructive Sleep Apnoea

Levels of proinflammatory cytokines, including IL-6, follow a circadian rhythm, suggesting a physiological role of these cytokines in sleep (Dominguez Rodriguez *et al.*, 2003). In OSA, intermittent hypoxia acts as a danger signal for the immune system and induces the synthesis of inflammatory cytokines (Kent *et al.*, 2011).

Previously our group found that 4 weeks of active CPAP treatment led to no significant change in inflammatory cytokines, IL-6 and IFN-γ, despite large improvements in both symptoms and objective sleepiness scores (Kohler *et al.*, 2009). However, other studies have found a positive correlation between CRP and proinflammatory cytokines, such as IL-6 and IL-18, with the severity of OSA (Minoguchi *et al.*, 2005; Shamsuzzaman *et al.*, 2002). Plasma levels of IL-6 and TNF-α were significantly higher in OSA patients compared to controls, and they both correlated with AHI (Ciftci *et al.*, 2004). Also TNF-α has also been reported to return to healthy control levels, following 6 weeks of CPAP therapy (Ryan *et al.*, 2005). Significantly lower IL-10 plasma levels have been detected in OSA patients compared to age and sex matched controls (Alberti *et al.*, 2003), suggesting a reduced regulation of inflammation.

The two double-blind randomised controlled studies in this Chapter, will also examine the effect of CPAP treatment on levels of CRP, IL-6, TNF-α and IL-10 in plasma.

6.1.6. Aims of this Chapter

1) To establish the impact of a 6 month introduction of CPAP therapy in minimally symptomatic OSA patients on circulating MPs and other inflammatory markers.

2) To determine the impact of a 2 week withdrawal from CPAP therapy in moderate to severe OSA patients on circulating MP levels and other inflammatory markers.

6.2. Methods

6.2.1. Patients and Trial Design

6.2.1.1. Effect of 6 months CPAP Treatment in Minimally Symptomatic OSA

The MOSAIC trial (Multicentre Obstructive Sleep Apnoea Interventional Cardiovascular trial) recruited patients with minimally symptomatic OSA. Patients were randomised to either 6 months use of CPAP therapy or 6 months supportive care. 54 patients had their MP levels assessed at the 0 and 6 month time points. The trial was approved by the Oxford research ethics committee (RECNo: 05/Q1604/159) and registered (ISRCTN 34164388). Written informed consent was obtained from all participants.

Patients were referred to the Oxford Centre for Respiratory Medicine. Patients were eligible for the trial if they were aged between 45 and 75 years, had proven OSA with a severity defined as >7.5 oxygen desaturations of >4 % per hour, and no history of excessive daytime sleepiness or other daytime symptoms of OSA which would have justified initiation of CPAP therapy. Patients with ventilatory failure, previous exposure to CPAP, blood pressure >180/110 mmHg, a current professional driver or a history of any sleep related accident, were excluded.

Patients randomised to CPAP therapy were instructed in the use of an auto-adjusting CPAP machine (Autoset S8, ResMed, Abingdon, UK) by a trained sleep nurse. Patients randomised to standard care were advised to continue with their current medication, but were not given specific weight reduction or lifestyle advice.

6.2.1.2. Effect of 2 weeks CPAP Treatment Withdrawal in Moderate/Severe OSA Patients

Patients previously diagnosed with OSA and treated with CPAP, were eligible for the trial if they were aged between 20 and 75 years, had an oxygen desaturation index (ODI, ≥4 % dips) of more than 10 per hour in their initial sleep study, and if they had been treated with CPAP for more than 12 months with a minimal average compliance of 4 hours per night. Patients previously diagnosed with unstable and untreated CAD, severe and inadequately controlled arterial hypertension, a history of any sleep related accident or who were current professional drivers were excluded from the study. The trial was approved by the University Hospital of Zurich research ethics committee (EK-1600) and registered (ISRCTN 93153804). Written informed consent was obtained from all participants.

The persistence of OSA was confirmed by home overnight pulse-oximetry on the last night of a 4-night period without CPAP, eligible patients returned to therapy with CPAP for at least one week. Polysomnographic sleep studies were performed and sleepiness was assessed using the Epworth sleepiness score (Johns, 1991).

41 patients were randomised to either continue with CPAP therapy for 2 weeks or switch to subtherapeutic CPAP for 2 weeks. In patients randomised to subtherapeutic CPAP, subtherapeutic pressure was achieved by setting the CPAP machine to the lowest pressure, inserting a flow-restricting connector at the machine outlet, and inserting six extra holes in the collar of the main tubing as previously described (Pepperell *et al.*, 2002).

6.2.2. Measurement of Microparticles

MPs were measured, blind of the patient group, by the assay described in the Chapter 2. Briefly, blood was drawn from fasting participants in the morning between 09.00 and 10.00 am. Within 15 mins of venepuncture, the tubes were centrifuged at 1550 g for 20 min, to produce PPP. 250 μl of PPP was frozen immediately and stored at -80 °C. The 250 μl PPP aliquots were thawed on ice and then washed twice at 18,000 g for 30 min.

In both studies, annexin V-FITC was used to stain procoagulant MPs. CD31-PE and CD41-PECy5 were used to differentiate between PMPs (CD31+CD41+) and EMPs (CD31+CD41-). CD144-PE and CD106-PE-Cy5 were also used as markers for EMPs. CD45-APC was used as a marker for LMPs. For the Zurich study, additional MP markers were used; lactadherin-FITC, CD62E-PE Cy5, CD66B-FITC, CD14-PE, CD3-PerCP and glycophorin-A-PE.

6.2.3. Measurement of Other Inflammatory Markers

For the MOSAIC study, heparin blood was drawn from fasting participants at baseline and 6 months later. For the Zurich study, heparin blood was drawn from patients at baseline and at 2 weeks. Measurements of hsCRP, IL-6, IL-10 and TNF-α were performed on plasma samples which had been stored at -80 °C.

IL-6, TNF-α and IL-10 were measured by high sensitivity ELISA using commercially available kits (BMS213HS, BMS223HS and BMS215HS, Bender MedSystems GmbH, Vienna, Austria). The lower limits of detection and the intra and inter-assay coefficients of variation are as described in Chapter 5. Cytokine measurements were carried out in duplicate and the mean value was reported.

CRP was analysed by the Siemens BN II nephelometry method, which measures a range between 0.18 – 1150 mg/l (Siemens, Surrey, UK).

6.2.4. Statistics

Statistics were performed using GraphPad Prism 5 (GraphPad Software, San Diego). Differences in baseline characteristics were assessed by independent t-tests and chi^2 tests as appropriate. Wilcoxon matched pairs tests were performed to assess within group changes. Comparisons of changes between groups were assessed by Mann-Whitney U tests, and expressed as median difference in change of levels between groups. Non-parametric confidence intervals were calculated using Confidence Interval Analysis Software (CIA Version 1.1). Associations between levels of MPs and other inflammatory markers in the CPAP Withdrawal Study were assessed by Spearman correlation. For correlations, results from all biomarkers were log transformed to normalise data. A p-value of <0.05 was considered statistically significant.

6.3. Results

6.3.1. Effect of 6 months CPAP Treatment in Minimally Symptomatic Obstructive Sleep Apnoea (MOSAIC)

6.3.1.1. Subject Characteristics

54 OSA patients were eligible for the study, 27 were randomly assigned to receive 6 months CPAP treatment and 27 were assigned to receive only supportive care during this time.

Table 6.1 shows the baseline characteristics of the two groups. There were no significant differences in the baseline characteristics.

Table 6.1 – Baseline Characteristics of Patient Groups

	CPAP Group (n=27)	Supportive Care Group (n=27)	P-value
Age	56.3 (6.3)	57.1 (6.0)	0.6152
Males/Females	25/2	26/1	0.6463
BMI	31.6 (5.5)	32.6 (5.3)	0.5387
Neck Circumference in cm	42.9 (3.7)	44.0 (3.5)	0.2549
Current Smokers (%)	14.8	18.5	0.7212
Ex Smokers (%)	40.7	29.6	0.4022
ESS before therapy	8.7 (3.9)	7.9 (3.7)	0.3958
Systolic BP	129.1 (8.2)	132.2 (12.7)	0.2886
Diastolic BP	82.1 (7.4)	82.5 (8.1)	0.8615

Values are mean (SD), unless otherwise stated

6.3.1.2. Procoagulant Microparticles

The levels of annexin V+ MPs were significantly decreased in the CPAP group (p=0.005), but not in the control group. The percentage decrease was greater in the CPAP treated group (-25.6 %) than the control group (-12.8 %) (Figure 6.2).

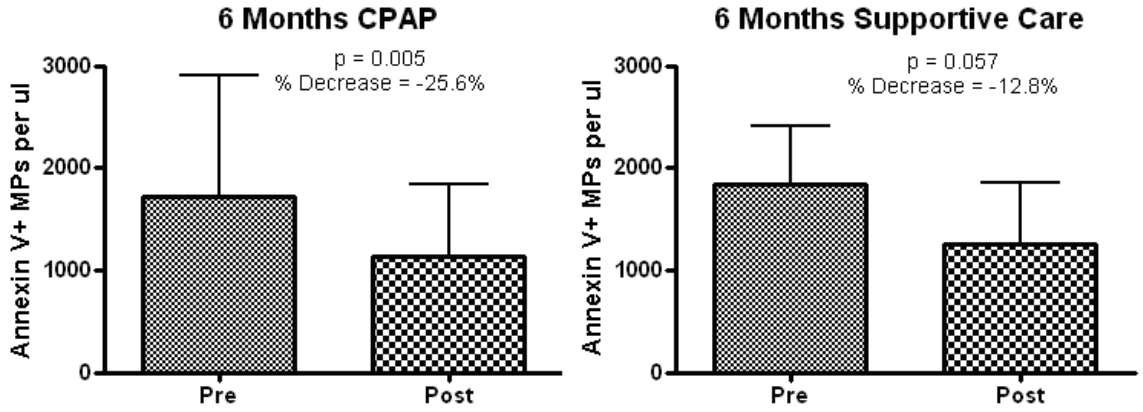

Figure 6.2 – Median (IQR) levels of annexin V+ MPs in OSA patients receiving either 6 months CPAP treatment or 6 months supportive care.

6.3.1.3. Platelet-Derived Microparticles

The levels of CD31+CD41+ PMPs were significantly decreased for both the CPAP group (p=0.008) and control group (p=0.029). However, the percentage decrease was greater in the CPAP treated group (-22.9 % versus -15.6 %) (Figure 6.3).

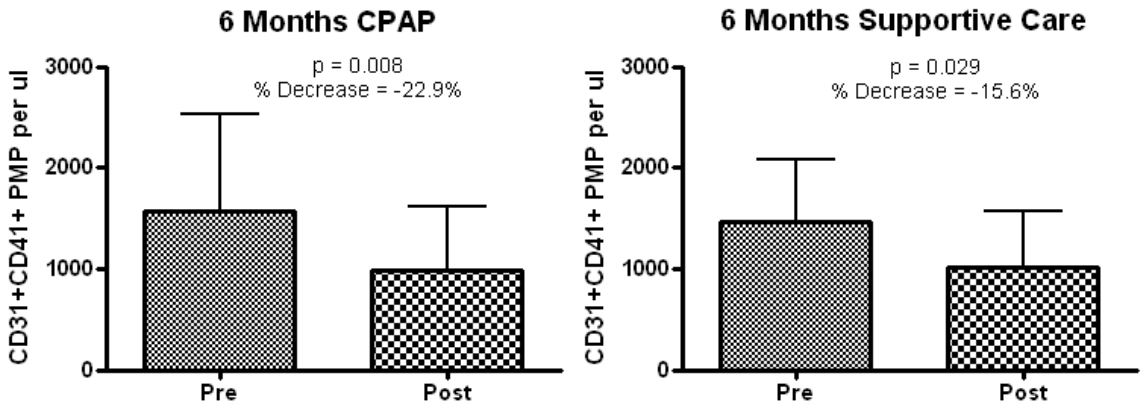

Figure 6.3 – Median (IQR) levels of CD31+CD41+ PMPs in OSA patients receiving either 6 months CPAP treatment or 6 months supportive care.

6.3.1.4. Leucocyte-Derived Microparticles

The levels of CD45+ LMPs were significantly increased for the CPAP group (p=0.007), but not for the control group (Figure 6.4).

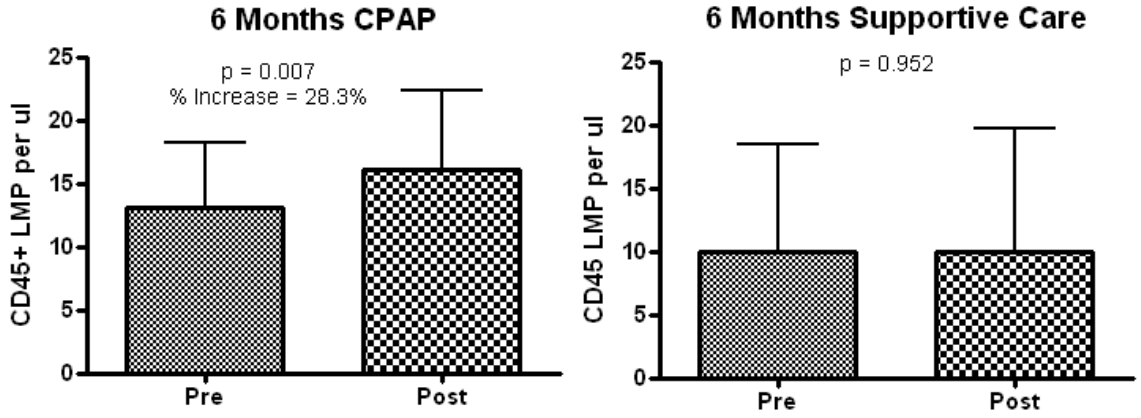

Figure 6.4 – Median (IQR) levels of CD45+ LMPs in OSA patients receiving either 6 months CPAP treatment or 6 months supportive care.

6.3.1.5. Endothelial Cell-Derived Microparticles

Six months of CPAP treatment did not significantly change the levels of CD31+CD41-, CD144+ and CD106+ EMPs in either of the groups (data not shown).

6.3.1.6. Effect of 6 Months CPAP Therapy on Levels of Inflammatory Markers

Table 6.2 – Effect of 6 Months CPAP Therapy on Levels of Inflammatory Markers

	Baseline Levels		Change from Baseline		
	CPAP Group	Supportive Care Group	CPAP Group	Supportive Care Group	P-value
CRP (mg/l)	1.89 (1.06 – 2.33)	2.21 (0.84 – 5.50)	0.01 (-1.16 – 0.22)	-0.15 (-1.76 – 1.10)	0.7819
IL-6 (pg/ml)	0.43 (0.22 – 0.58)	0.48 (0.16 – 1.03)	0.00 (-0.19 – 0.22)	0.04 (-0.16 – 0.23)	0.5165
TNF-α (pg/ml)	1.31 (1.01 – 1.87)	1.31 (1.03 – 1.61)	0.20 (-0.08 – 0.32)	0.14 (-0.13 – 0.23)	0.7489
IL-10 (pg/ml)	0.68 (0.40 – 1.10)	0.50 (0.25 – 0.75)	0.02 (-0.11 – 0.23)	0.09 (-0.08 – 0.32)	0.6843

Values are median (interquartile range)

There was no significant change from baseline values in CRP, IL-6, TNF-α, or IL10 in either the CPAP or the supportive care group.

6.3.2. Effect of 2 weeks CPAP Treatment Withdrawal in Moderate to Severe OSA Patients (Zurich Study)

6.3.2.1. Subject Characteristics

41 patients were eligible for the study. 20 subjects were randomly assigned to continue on therapeutic CPAP and 21 subjects were randomly assigned to receive subtherapeutic CPAP. One patient from the subtherapeutic CPAP group withdrew after randomisation and two patients from the subtherapeutic CPAP group could not have blood drawn at two weeks (Figure 6.5). The baseline patient characteristics are shown in Table 6.3.

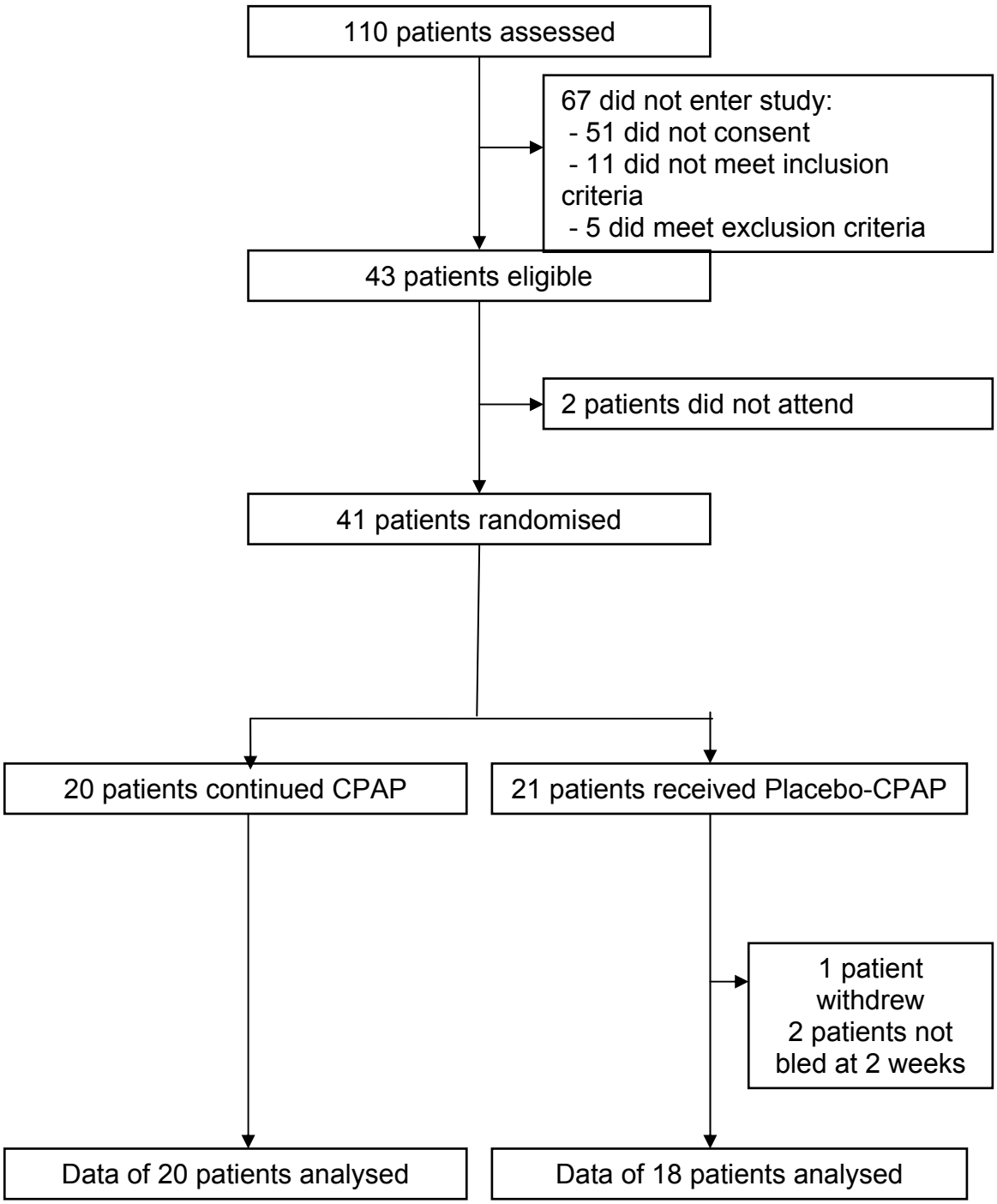

Figure 6.5 – Trial Profile for Effect of 2 weeks CPAP Treatment Withdrawal in Moderate/Severe OSA Patients

Table 6.3 – Baseline Patient Characteristics

	Therapeutic CPAP group (n=20)	Subtherapeutic-CPAP group (n=18)	P value
Age	63.6 (5.1)	61.8 (7.5)	0.393
Males/females	19/1	21/0	0.300
BMI	32.9 (6.5)	33.1 (4.4)	0.904
Waist/hip circumference ratio	1.0 (0.1)	1.0 (0.0)	0.106
Neck circumference in cm	46.4 (3.7)	46.1 (4.2)	0.838
Current smokers (%)	19.1	5.0	0.170
Ex-smokers (%)	38.1	25.0	0.368
Hypertension (%)	80.9	70.0	0.414
Diabetes (%)	23.8	20.0	0.768
CAD (%)	4.8	10.0	0.520
Cholesterol-lowering medication (%)	33.3	35.0	0.910
Systolic blood pressure	133.3 (16.6)	129.2 (12.7)	0.380
Diastolic blood pressure	82.3 (7.8)	82.2 (8.2)	0.980
AHI original sleep study	36.0 (17.3)	45.3 (22.3)	0.155
ODI original sleep study	26.6 (13.5)	37.3 (22.7)	0.141
AHI on CPAP	5.1 (2.7)	4.3 (2.3)	0.329
CPAP compliance in min	373.1 (67.9)	362.8 (72.3)	0.642
ESS before therapy	13.8 (2.6)	15.3 (3.5)	0.188
ESS on CPAP	7.4 (3.1)	6.6 (2.7)	0.399

Values are means (SD), unless otherwise stated.

There were no significant differences between the baseline characteristics of the two patient groups.

The effectiveness of CPAP withdrawal for the return of OSA symptoms was examined by measuring the AHI, ODI and ESS at baseline and after 2 weeks in both patient groups (Table 6.4).

Table 6.4 – Effect of CPAP-Withdrawal on Sleep Disordered Breathing and Sleepiness

	Baseline		Change from baseline		
	Therapeutic CPAP (n=20)	**Subtherapeutic CPAP (n=18)**	**Therapeutic CPAP (n=20)**	**Subtherapeutic CPAP (n=18)**	**P value**
AHI (events/h)	1.7 (1.8)	2.2 (2.5)	+0.4 (2.8)	+33.8 (24.3)	<0.001
ODI (events/h)	0.5 (0.8)	0.9 (2.0)	-0.2 (1.0)	+26.3 (22.9)	<0.001
ESS	7.4 (3.1)	6.6 (2.7)	-0.7 (2.2)	+2.0 (2.7)	0.001

Values are means (SD). P-value is Mann Whitney test between Change from Baseline in Therapeutic CPAP versus Subtherapeutic CPAP

Withdrawal of CPAP therapy led to a significant increase in AHI and ODI at 2 weeks, in comparison to continuation on CPAP ($p<0.001$). ESS increased significantly at 2 weeks in the subtherapeutic group compared to the therapeutic group ($p=0.001$). These results indicate a return of OSA symptoms in the subtherapeutic group after 2 weeks.

6.3.2.2. Procoagulant Microparticles

Withdrawal of CPAP therapy did not significantly change the number of annexin V+ MPs or lactadherin+ MPs, although there was a trend for them to increase in the Subtherapeutic CPAP group (Figure 6.6).

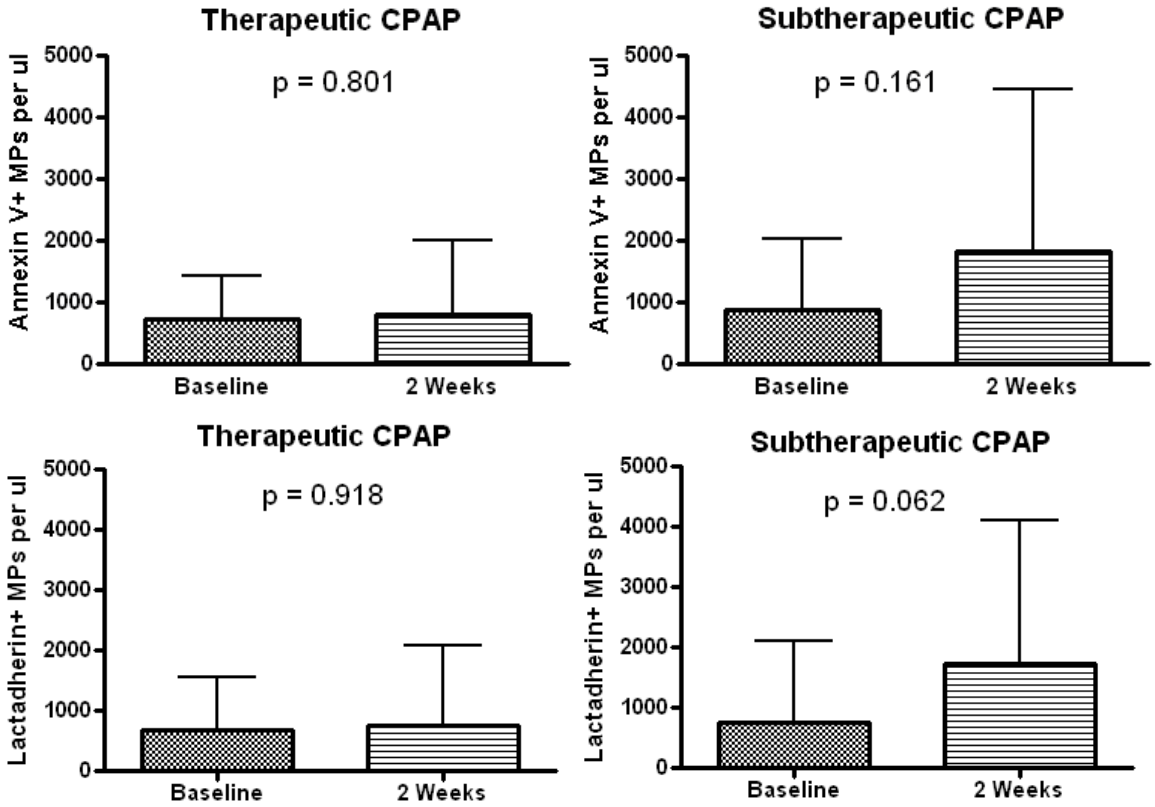

Figure 6.6 – Median (IQR) levels of annexin V+ and lactadherin+ MPs in OSA patients receiving either therapeutic or subtherapeutic CPAP

6.3.2.3. Platelet-derived Microparticles

Withdrawal of CPAP therapy did not significantly change the number of CD31+CD41+ PMPs in either group.

6.3.2.4. Leucocyte-derived Microparticles

Withdrawal of CPAP therapy did not significantly change the number of CD45+ LMPs in either group.

6.3.2.5. RBC-Derived Microparticles

Withdrawal of CPAP therapy did not significantly change the number of glycophorin A+ RBC MPs in either group

6.3.2.6. Endothelium-derived Microparticles

Withdrawal of CPAP therapy was associated with a significant increase in the level of both CD62E+ EMPs (p=0.040) and CD106+ EMPs (p=0.007), not seen in the therapeutic CPAP group (Figures 6.7 and 6.8). There was also a significant difference in the change in CD62E+ EMP levels (median difference in change +32.4 per µl (95 % CI +7.3 to +64.1 per µl), p=0.010) (Figure 6.7), but not in CD106+ EMPs (median difference in change +21.9 per µl (95 % CI -4.7 to +166.0 per µl) p=0.105), between the therapeutic and subtherapeutic groups.

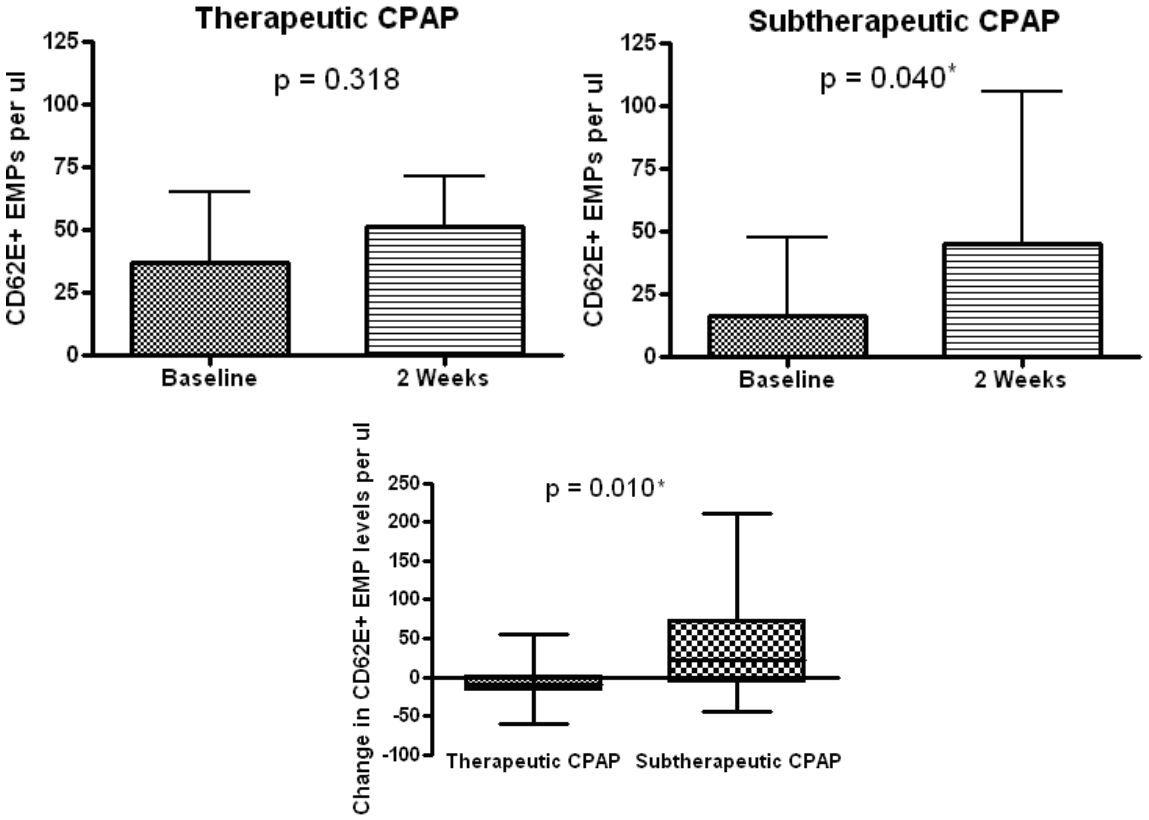

Figure 6.7 – Median (IQR) levels of CD62E+ EMPs in OSA patients receiving either therapeutic or subtherapeutic CPAP. Box and whisker plot shows change in CD62E+ EMPs between baseline and 2 weeks

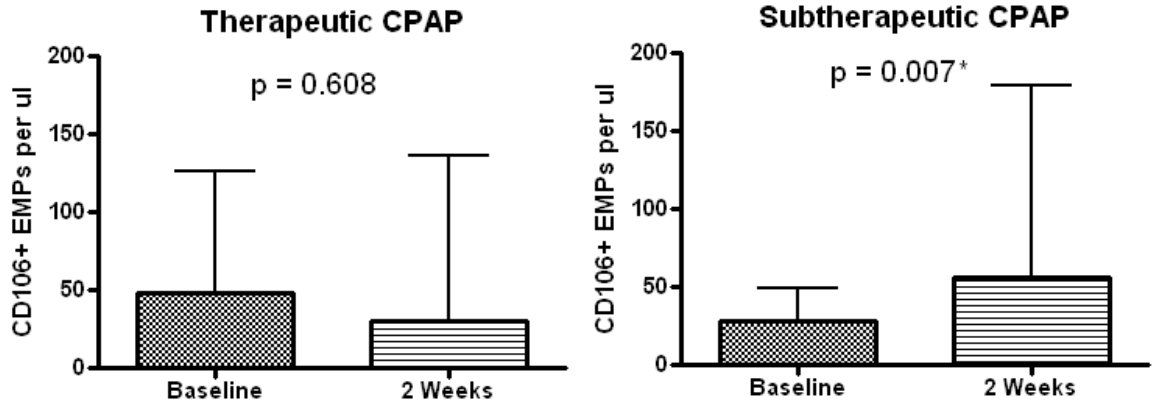

Figure 6.8 – Median (IQR) levels of CD106+ EMPs in OSA patients receiving either therapeutic or subtherapeutic CPAP.

Withdrawal of CPAP therapy did not significantly change the levels of CD31+CD41- EMPs or CD144+ EMPs in either group.

6.3.2.7. Granulocyte-derived Microparticles

Withdrawal of CPAP therapy was associated with a significant increase in the number of CD66B+ granulocyte-derived MPs (p=0.029), not seen in the therapeutic CPAP group (Figure 6.9). Although, there was no significant difference in the change in CD66B+ MP levels between the therapeutic and subtherapeutic groups (median difference in change +16.3 per µl (95 % CI -5.3 to +43.8 per µl) p=0.132).

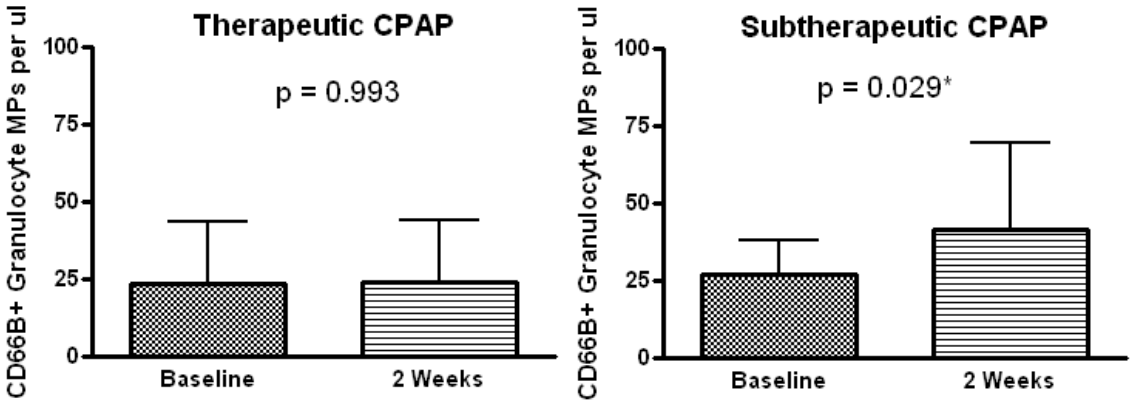

Figure 6.9 – Median (IQR) levels of CD66B+ granulocyte-derived MPs in OSA patients receiving either therapeutic or subtherapeutic CPAP

6.3.2.8. Monocyte-derived Microparticles

Withdrawal of CPAP therapy was associated with a significant increase in the number of CD14+ monocyte-derived MPs (p=0.002), not seen in the therapeutic CPAP group (Figure 6.10). Although, there was no significant difference in the change in CD14+ MP levels between the therapeutic and subtherapeutic groups (median difference in change +15.6 per µl (95 % CI -10.4 to +58.0 per µl) p=0.193).

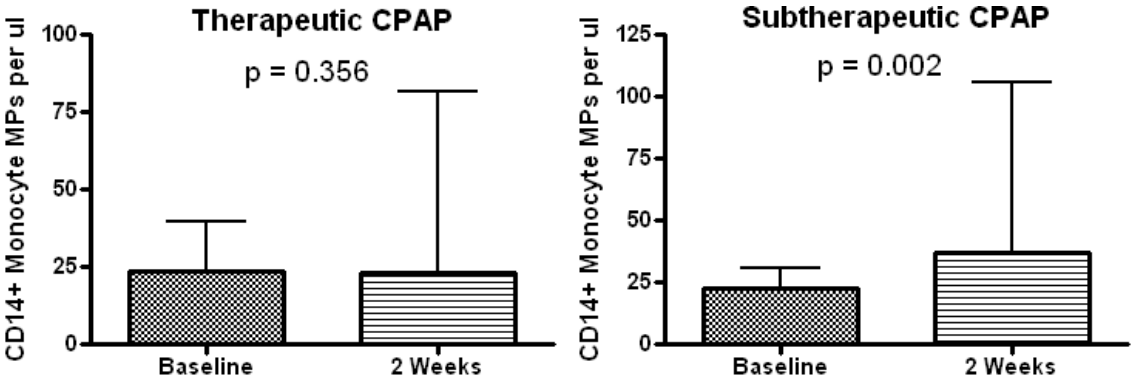

Figure 6.10 – Median (IQR) levels of CD14+ monocyte-derived MPs in OSA patients receiving either therapeutic or subtherapeutic CPAP

6.3.2.9. Effect of CPAP Withdrawal on Levels of Inflammatory Markers

Table 6.5 – Effect of CPAP Withdrawal on Markers of Inflammation

	Baseline		Change from baseline		
	Therapeutic CPAP (n=20)	**Subtherapeutic CPAP (n=21)**	**Therapeutic CPAP (n=20)**	**Subtherapeutic CPAP (n=18)**	**P value**
CRP (mg/ml)	1.2 (0.7 – 2.4)	2.4 (1.0 – 4.5)	-0.1 (-0.3 – 0.6)	-0.3 (-1.0 – 0.1)	0.077
IL-6 (pg/ml)	0.5 (0.3 – 1.0)	0.3 (0.3 – 0.8)	0.0 (-0.2 – 0.2)	0.0 (-0.2 – 0.1)	0.838
TNF-α (pg/ml)	0.7 (0.5 – 0.7)	0.6 (0.5 – 0.7)	0.0 (-0.1 – 0.1)	0.0 (-0.1 – 0.1)	0.661
IL-10 (pg/ml)	0.8 (0.5 – 1.3)	0.6 (0.5 – 1.1)	0.0 (-0.2 – 0.1)	0.0 (-0.1 – 0.2)	0.413

Values are medians (quartiles). P-value is Mann Whitney test between Change from Baseline in Therapeutic CPAP versus Subtherapeutic CPAP

The effect of CPAP withdrawal on other markers of inflammation was also assessed. There were no significant changes in CRP, IL-6, IL-10 or TNF-α at 2 weeks, in the subtherapeutic group compared to the therapeutic CPAP group. These findings have been published in the American Journal of Respiratory and Critical Care Medicine (Kohler *et al.*, 2011).

6.3.2.10. Associations between Microparticle and Other Inflammatory Marker Levels

To examine any link between MPs and other markers of inflammation, correlation analysis was performed. There were no significant associations found between IL-6, IL-10 and TNF-α and levels of any MP subtype (data not shown). CRP was found to be weakly positively associated with annexin V+ MPs, lactadherin+ MPs and CD31+CD41+ PMPs in the CPAP Withdrawal study (Figure 6.11).

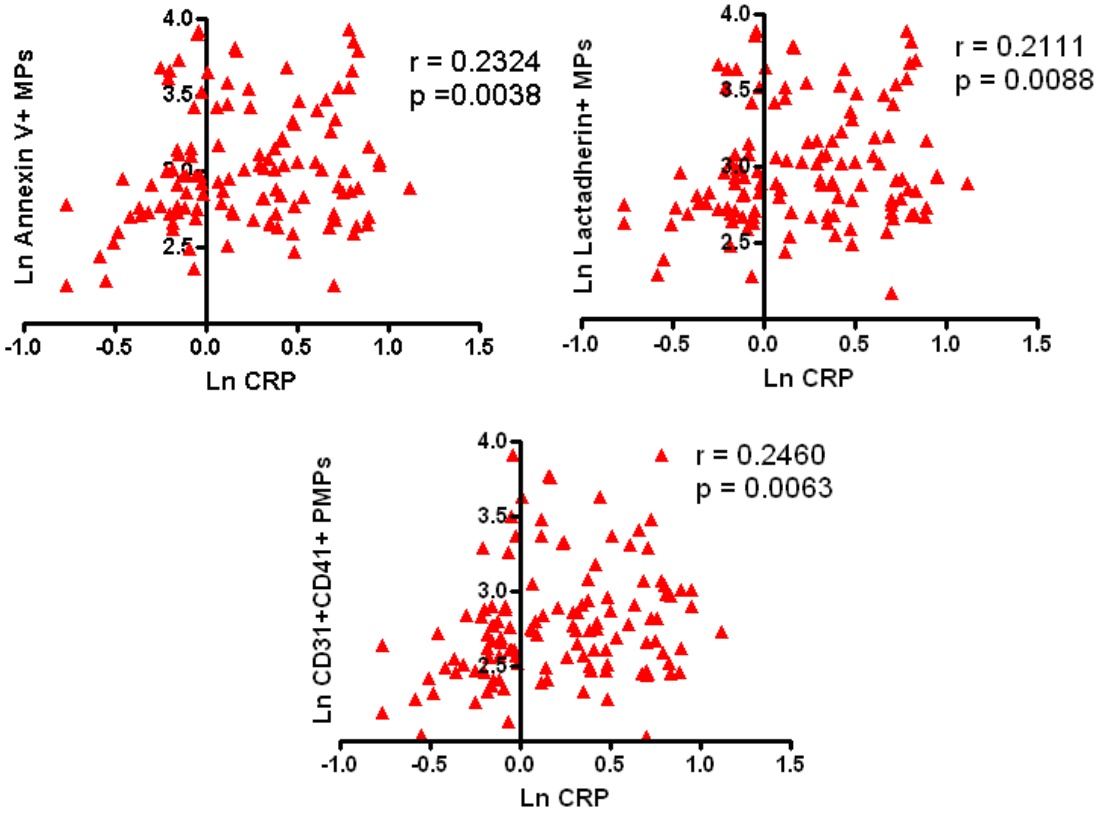

Figure 6.11 – Associations between CRP and proacoagulant MP and PMP Levels. Data was log transformed.

6.4. Discussion

6.4.1. Microparticles in Obstructive Sleep Apnoea

There have been no previous published reports of randomised controlled trials investigating the effects of CPAP therapy on the level of MPs. The first trial in this Chapter showed that annexin V+ MPs were significantly reduced in a group of minimally symptomatic OSA patients receiving 6 months CPAP therapy, and not changed in the group receiving only supportive care. PMP levels were significantly reduced in both groups of patients, and levels of LMPs were significantly elevated following 6 months of CPAP treatment, but were not altered in the control group. Levels of EMPs were not changed in either group of patients.

The second trial showed that withdrawal of CPAP therapy for 2 weeks was associated with a significant increase in CD62E+ and CD106+ EMP, granuolocyte-derived MP and monocyte-derived MP levels in moderate/severe OSA patients, not seen in the those patients continuing therapy.

OSA has been shown to be an independent causal factor for hypertension, and a risk factor for other cardiovascular diseases (Marin *et al.*, 2005; Peker *et al.*, 2006). The current opinion is that the consequences of OSA, including intermittent hypoxia, intrathoracic pressure changes and arousals, may result in inflammation, endothelial dysfunction and ultimately arterial disease (Kohler and Stradling, 2010).

MPs could provide a linking mechanism between OSA and CVD. MPs appear to be both a cause and a consequence of endothelial dysfunction, and their levels can be correlated with other markers of endothelial dysfunction (Amabile *et al.*, 2005). OSA is considered to represent a proinflammatory state, demonstrated by increased levels of proinflammatory proteins and cytokines (Minoguchi *et al.*, 2005; Shamsuzzaman *et al.*, 2002). MPs may be produced in response to inflammation, but they also have potent proinflammatory potential. MPs can also express tissue factor, giving them thrombotic properties. Therefore, MPs have the potential to increase cardiovascular risk in OSA patients, through endothelial dysfunction, inflammation and thrombosis.

6.4.1.1. Procoagulant Microparticles

In the first trial, a significant reduction of annexin V+ MPs was found following 6 months of CPAP treatment. Procoagulant MPs are produced in response to a variety of stimuli, including hypoxia, inflammatory cytokines and shear stress (Nomura *et al.*, 2008). CPAP treatment has been shown to reduce hypoxia, surges in blood pressure and levels of inflammatory cytokines (Lattimore *et al.*, 2006; Steiropoulos *et al.*, 2009). Therefore, CPAP treatment may be directly responsible for this reduction (Figure 6.12).

In the second trial, withdrawal of CPAP therapy did not lead to a significant increase in the number of total procoagulant MPs, determined by annexin V and lactadherin. The majority of circulating procoagulant MPs are platelet-derived, and as PMPs were not significantly altered by CPAP withdrawal, it would be unlikely to detect a significant change in procoagulant MPs.

6.4.1.2. Platelet-derived Microparticles

PMP levels have previously been shown to be elevated in patients with minimally symptomatic OSA (Ayers *et al.*, 2009). Therefore, it may be expected that a treatment, which significantly improves the pathological consequences and symptoms of OSA, could also reduce the levels of PMPs.

A recent study found that PMP levels were elevated in severe OSA and that PMP levels were correlated with AHI. 6 months CPAP treatment led to a significant reduction in PMP levels (Maruyama *et al.*, 2012b). However, they only examined the effect of CPAP therapy in 7 patients, and they used an ELISA based method for the detection of PMPs.

In the first trial, a significant reduction of PMPs was found in both the CPAP treated patients and those receiving just supportive care. It is possible that the control group showed a placebo effect and whilst taking part the patients in the supportive care group reduced their cardiovascular risk factors and therefore their circulating levels of PMPs.

In the second trial, PMPs were not significantly changed from baseline after either 2 weeks of therapeutic or subtherapeutic CPAP. It may be that 2 weeks CPAP withdrawal was not sufficient to cause a significant increase in the levels of PMPs.

6.4.1.3. Endothelial cell-derived Microparticles

The first trial found no significant alterations in CD31+CD41- EMPs, CD144+ and CD106+ EMPs over the 6 months. This may be due to minimally symptomatic OSA patients having very small EMP populations, making it difficult to determine small changes in their levels.

It is recognised that different subtypes of EMPs are formed by different mechanisms. CD62E+ EMPs are suggested to be markers of early endothelial cell activation, whilst CD144+ and CD31+CD41- EMPs reflect structural damage of endothelial cells (Amabile *et al.*, 2008). CD62E+ EMPs were not measured in the first trial, and this may explain why a significant reduction in EMPs was not found.

In the second trial, CD62E+ EMPs and CD106+ EMPs were both significantly increased from baseline to 2 weeks in the subtherapeutic group, and the change in CD62E+ EMP levels was significantly different between the therapeutic and the subtherapeutic groups (Figure 6.12). CD31+CD41- EMPs and CD144+ EMPs did not significantly change over the two weeks in either patient group.

Yun *et al* (2010) reported significantly higher levels of CD31+CD42- EMPs and CD62E+ in OSA than in matched controls, and that CD31+CD42- EMPs were correlated with OSA severity. Following CPAP treatment, a significant reduction in CD62E+ EMPs but not CD31+CD42- EMPs was found, supporting the findings in this Chapter. CPAP treatment has previously been shown to improve endothelial function (Cross *et al.*, 2008; Ip *et al.*, 2004), and the decrease in EMP levels may be an underlying mechanism of endothelial function improvement. However, it is remains unclear as to whether EMPs are a cause or a consequence of endothelial dyfunction.

6.4.1.4. Leucocyte-derived Microparticles

LMP levels have been previously shown to be elevated in patients with minimally symptomatic OSA (Ayers *et al.*, 2009; Priou *et al.*, 2010).

In the first trial, a significant increase in LMP levels was found in the CPAP-treated group, but not in the control group. This increase was unexpected, as levels of other MPs appear to be reduced following CPAP, and may suggest that different MP subtypes are triggered by very different mechanisms. Kim *et al* (2011) found that LMPs were positively associated with AHI, and as CPAP treatment is known to lower AHI, it was expected that LMPs would also be lowered. However, the decrease in procoagulant MPs alongside the increase in LMPs may suggest a skewing from a procoagulant role, to one involving inflammation.

In the second trial, withdrawal of CPAP treatment was not associated with a significant change in the number of CD45+ LMP levels. It is possible that 2 weeks of CPAP withdrawal was not sufficient to cause a significant increase in LMPs.

6.4.1.5. Granulocyte-Derived Microparticles

Withdrawal of CPAP therapy was associated with a significant increase in the number of granulocyte-derived MPs (Figure 6.12). A previous study found that granulocyte-derived MPs were increased in OSA patients, with an ODI of ≥10 events per hour (Priou *et al.*, 2010). Polymorphonuclear cells have an enhanced readiness to respond with superoxide generation in OSA, which is reversed by CPAP treatment (Schulz *et al.*, 2000). Therefore, it may be expected that granulocyte-derived MPs, a marker of polymorphonuclear cell activation, would be significantly elevated following withdrawal from CPAP.

6.4.1.6. Monocyte-Derived Microparticles

Withdrawal of CPAP therapy was associated with a significant increase in monocyte-derived MPs in the CPAP withdrawal group, not seen in the continuing CPAP group. This is the first study to have analysed the levels of monocyte-derived MPs in OSA patients. Monocyte MPs can cause activation of the endothelium, by delivering IL-1β (Wang *et al.*, 2011), and therefore they may be another trigger of endothelial dysfunction in OSA.

6.4.1.7. Red Blood Cell-Derived Microparticles

Withdrawal of CPAP was not associated with a significant change in the number of RBC-derived MPs, suggesting that they are not a major subset of MPs affected by CPAP treatment in OSA patients.

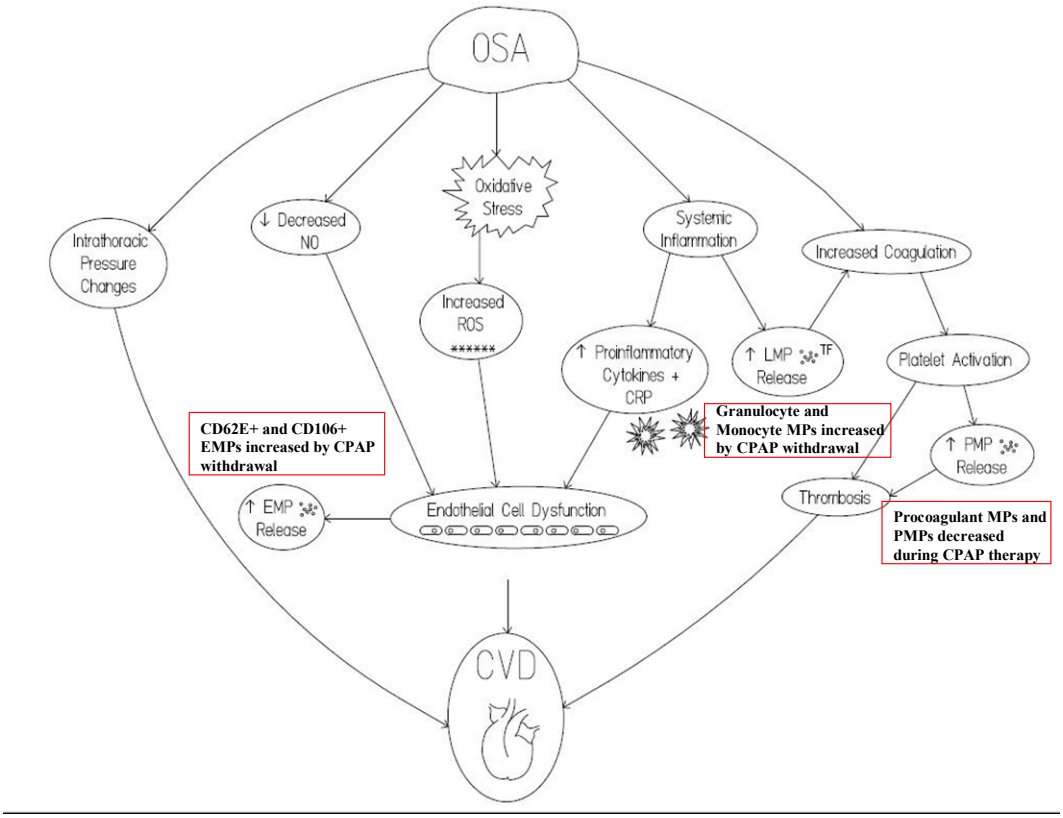

Figure 6.12 – Possible mechanisms linking obstructive sleep apnoea and cardiovascular disease, including findings from randomised controlled trials.

6.4.2. Other Inflammatory Markers in Obstructive Sleep Apnoea

There is evidence from *in vitro* and controlled studies in humans, implicating systemic inflammation as an important mechanism linking OSA with endothelial dysfunction and vascular disease, but there is little data from randomised controlled trials on this possible association.

The first trial found that there were no significant changes in the levels of CRP, IL-6, TNF-α or IL-10, following introduction of CPAP therapy for 6 months. This is corroborated by previous trials, including two which both found that 3 months of CPAP therapy did not alter levels of IL-6 and TNF-α in OSA patients (Arias *et al.*, 2008; Vgontzas *et al.*, 2008).

The second trial in this Chapter found that 2 weeks of CPAP withdrawal did not lead to a change in cytokines, IL-6, TNF-α and IL-10. These findings confirm the results of an uncontrolled study in which no significant increases in IL-6 and TNF-α after 7 days of CPAP withdrawal were found (Phillips *et al.*, 2007).

2 weeks of CPAP withdrawal did not cause a significant change in levels of CRP. This is supported by a study which also found no change in CRP levels following 7 days of CPAP withdrawal (Phillips *et al.*, 2007). However, another study found that treatment for three months with both fixed and auto-adjusting CPAP did cause a significant decrease in CRP (Patruno *et al.*, 2007).

CPAP withdrawal for two weeks, in this Chapter, led to the recurrence of OSA and an increase in sleepiness score, so is an effective for examining a return of OSA. However, it may be that 2 weeks of CPAP withdrawal was not sufficient to induce a systemic inflammatory response.

6.4.2.1. Associations between Microparticle and Other Inflammatory Marker Levels

IL-6 was not significantly associated with levels of any subtype of MPs, confirming previous findings of no association between IL-6 and PMPs (Diamant *et al.*, 2002). However, significant associations have been found between PMPs and IL-6 in a study of 464 healthy individuals (Ueba *et al.*, 2010a). Data in this Chapter reveal no association between TNF-α and any MP subtype, in contrast to Diamant *et al* (2002) who noted a positive association between levels of PMPs and TNF-α when measuring them in type 2 diabetes patients and healthy controls. There was also no association between IL-10 and MP levels. IL-10 is an anti-inflammatory cytokine, so it may have been expected to be inversely correlated with proinflammatory MPs.

The lack of associations between the cytokines measured here and the MPs could be explained by the very low levels of cytokines detected. Discrepancies between other studies that have examined the associations of MPs with these cytokines, may be due to the subjects selected and the techniques used to measure MPs.

CRP was weakly positively associated with annexin V+ MPs, lactadherin+ MPs and PMPs. This is supported by Ueba *et al* (2010) who found a positive association between CRP and PMPs in 210 healthy men. CRP itself can be found on the surface of MPs, and CRP+ MPs were found to be positively associated with CRP levels in acute myocardial infarction patients (van der Zee *et al.*, 2010). The fact that the associations were found to be weak in this Chapter, suggests that although MPs and CRP are in some way associated, their interactions are complex, and that there are many other factors which have an impact on their levels.

6.4.3. Limitations

The patients in the first trial had only minimally symptomatic OSA, and therefore their elevations of MPs were small, making it difficult to detect subtle changes in levels following treatment.

In the second trial, a longer CPAP withdrawal period may have been associated with more pronounced changes in MP levels. However, because of ethical issues, it would be difficult to prolong this intervention time.

In both studies, samples were only centrifuged once at 1550 g for 20 minutes, to remove cells and platelets, prior to freezing. However, all samples were processed within 15 minutes and were all handled identically.

6.4.4. Conclusions

These are the first randomised controlled trials to assess the impact of CPAP therapy on MP levels. Treatment for 6 months with CPAP appears to reduce levels of annexin V+ MPs in minimally symptomatic patients. This suggests that CPAP treatment reduces some of the stimuli that trigger release of these MPs. However, LMPs were elevated following CPAP treatment, suggesting that different mechanisms trigger the release of distinct MP subpopulations.

Withdrawal of CPAP therapy for 2 weeks, in moderate to severe OSA patients, was associated with a significant increase in EMPs, granulocyte-derived MPs and monocyte-derived MPs. These results provide some evidence that MP formation may be causally linked to OSA and may promote endothelial activation in these patients.

Chapter 7 – Microparticles in Patients with Increased Risk of Cardiovascular Disease

7.1. Introduction

7.1.1. Epidemiology of Cardiovascular Disease and Assessment of Cardiovascular Disease Risk

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the developed world (Girn *et al.*, 2007). It is the cause of almost 198,000 deaths each year in the UK, approximately half of which are due to coronary artery disease (CAD) (Allender *et al.*, 2008a). Death rates from CVD, specifically CAD, have been falling since the 1970s, due to reductions in the major risk factors, particularly smoking, and improvements in treatments. However, in certain groups, such as young women, this improvement in mortality appears to be declining (Allender *et al.*, 2008b).

Early detection of cardiovascular risk allows for modifications of lifestyle factors, reducing risk in individuals. Several risk score calculations have been developed to predict sub-clinical atherosclerosis, including the Framingham risk score and Systemic Coronary Risk Evaluation (Raiko *et al.*, 2010). A risk score takes into account certain risk factors, including age, sex, BP, cholesterol, smoker status and history of CVD, to predict the risk of a fatal cardiovascular event in the subsequent years (Pocock *et al.*, 2001).

There is considerable interest in blood biomarkers that can provide additional information about the status of the cardiovascular system at the early stages of atherosclerosis before actual clinical manifestations. Currently the blood biomarkers recommended for use in cardiovascular risk prediction are LDL cholesterol, HDL cholesterol and triglycerides. However it has been suggested that the measurement of inflammatory markers may enhance risk evaluation (Packard and Libby, 2008).

7.1.2. Coronary Artery Disease and Microparticles

CAD is the narrowing of the vessels which supply blood to the heart, due to plaques forming in the coronary arteries. Angina is the most common symptom, as well as shortness of breath and tiredness following exertion.

It is known that patients with CAD have increased levels of EMPs (Bernal-Mizrachi *et al.*, 2003; Koga *et al.*, 2005), and that annexin+CD31+ MPs are positively correlated with coronary endothelial dysfunction (Werner *et al.*, 2006). In CAD it is important to note that circulating MPs can be derived from two sources. Firstly they can originate from

circulating activated and apoptotic blood and endothelial cells. Secondly MPs can be shed from within an atherosclerotic plaque following plaque rupture (Morel *et al.*, 2006). Plaque MPs can be derived from macrophages, lymphocytes, RBCs, smooth muscle cells and endothelial cells (Leroyer *et al.*, 2007), and the composition differs from that normally found in the circulation. Significantly higher levels of procoagulant MPs, LMPs and CD105+ EMPs were found within the occluded coronary artery of patients following myocardial infarction, than in their peripheral blood (Morel *et al.*, 2009), suggesting that circulating peripheral levels of MPs do not necessarily indicate local levels.

7.1.3. Preeclampsia

Preeclampsia is defined as hypertension (diastolic BP ≥140 mm Hg on two or more occasions) and proteinuria (≥300 mg/24 hour urine) developed after 20 weeks of pregnancy in an otherwise normotensive woman (Valdiviezo *et al.*, 2012). It appears to be an inflammatory process, leading to an exaggerated systemic immune response to the pregnancy.

Normal pregnancy is associated with adaptations of the cardiovascular system, however in preeclampsia these adaptations are inadequate (VanWijk *et al.*, 2000). In normal pregnancy, endothelial cells, leucocytes and the coagulation system are all activated, but in preeclampsia this activation is exaggerated (Redman *et al.*, 1999).

Certain cell-specific MP subtypes are elevated in preeclampsia. For example, increased levels of placenta-derived MPs, have been reported in the circulation of women with a preeclamptic pregnancy compared to normotensive pregnant women (Germain *et al.*, 2007). Elevated T cell-derived and granulocyte-derived MPs (VanWijk *et al.*, 2002), as well as monocyte-derived and RBC-derived MPs (Lok *et al.*, 2008), have also been described in preeclampsia. Also increased numbers of CD31+CD42- EMPs were found in preeclampsia compared to controls (Gonzalez-Quintero *et al.*, 2003). MPs isolated from preeclamptic women can cause increased expression of ICAM-1 on endothelial cells co-cultured with monocytes *in vitro*, suggesting that they increase endothelial activation (Lok *et al.*, 2011).

Following a preeclamptic pregnancy women are at increased future risk of CVD (Valdiviezo *et al.*, 2012). In the 10-15 years after a preeclamptic pregnancy, there is an increased risk of hypertension, CAD and stroke (Bellamy *et al.*, 2007). The absolute risk for a cardiovascular event at age 50-59 years in women with a previous preeclamptic pregnancy is 17.8 %, compared to 8.3 % in women with no history of preeclampsia (Valdiviezo *et al.*, 2012). One hypothesis for this increased risk could be that abnormal levels of certain MP subtypes persist long after the preeclamptic pregnancy, increasing the

risk of CVD. Very few studies have investigated levels of MPs following a preeclamptic pregnancy.

7.1.4. Dobutamine Stress Echocardiogram

Dobutamine stress echocardiography (DSE) is a screening test for the evaluation of CAD. Dobutamine is a sympathomimetic drug that has direct interactions with α_1, β_1 and β_2 adrenergic receptors and induces cardiac stress by increasing contractility and cardiac output. During DSE, patients receive large doses of dobutamine, whilst their heart rate, BP and electrocardiogram (ECG) are measured. It is carried out in patients referred with chest pain and CVD risk factors, and is used to identify patients who would benefit from an angiogram, which is a more invasive confirmatory test. DSE is most effective at identifying coronary artery lesions which have caused at least 70 % narrowing of the artery (Ho *et al.*, 1995).

DSE was originally used in patients who could not carry out a maximum exercise echocardiogram due to disability or age (Dagianti *et al.*, 1995). However, the current NICE guidelines recommend the use of either exercise echocardiogram or DSE for imaging of stress induced cardiac wall motion abnormalities (Cooper *et al.*, 2010). The sensitivity and specificity of DSE for the detection of CAD are both >85 % (Sicari *et al.*, 2008). The measurement of certain inflammatory biomarkers in the plasma, such as IL-6, during the peak phase of DSE, provides additive prognostic value to this test for future cardiovascular events (Ikonomidis *et al.*, 2008).

One previous study found a trend for PMPs to be increased during and following DSE, although this did not reach statistical significance (Galloway *et al.*, 1998). Since that study, there have been no reports on the role of MPs during DSE. However, MPs have been shown to be elevated following strenuous exercise in healthy individuals (Chaar *et al.*, 2011; Maruyama *et al.*, 2012a), which mimics the physiological effects of DSE.

This chapter will examine the effect of DSE on MP levels before and immediately after DSE. It will also examine the use of MPs as a diagnostic marker by analysing the difference in MP levels between patients with a negative DSE (low chance of atherosclerosis) and those with a positive DSE (high chance of atherosclerosis).

7.1.5. Aims of this Chapter

1) To measure circulating MP levels in women 5-10 years following either a healthy or preeclamptic pregnancy, to determine if elevated levels of MPs persist after the pregnancy.

2) To determine the effect of a DSE on MP levels in patients with suspected atherosclerosis.

3) To identify any differences in MP levels between patients with a positive DSE compared to those with a negative DSE, in order to investigate if MPs are diagnostically useful in atherosclerosis.

7.2. Method

7.2.1. Preeclampsia Vascular Study

7.2.1.1. Subjects

Participants were identified from the John Radcliffe Hospital maternity records, and sent invitation letters for the study. The inclusion criteria for cases was females aged between 21-50 years who had experienced new onset hypertension and proteinuria during a previous pregnancy 5-10 years ago. The inclusion criteria for controls was females aged between 21-50 years who had a normal obstetric history, during a previous pregnancy 5-10 years ago. For both cases and controls the exclusion criteria was anyone who showed doubt in taking part, any pre-existing renal or cardiac disease, insulin dependent diabetes, or pre-existing hypertension. 76 cases and 46 controls were recruited. This study was approved by the Oxfordshire Research Ethics Committee (REC No. 08/H0604/127). All participants gave written informed consent.

7.2.1.2. Samples

Citrate blood samples were obtained from all participants. Within 15 min, citrate blood samples were centrifuged at 1550 g for 20 min, to produce PPP. 250 µl of PPP was taken, avoiding the buffy coat, and immediately frozen at -80 ºC.

7.2.2. Dobutamine Stress Echocardiogram Study

7.2.2.1. Subjects

51 participants were recruited from all patients referred to cardiology with a clinical indication for DSE testing. Patients were eligible for the study if they were greater than 18 years old and were able to give informed consent. Patients were excluded if they had a previous myocardial infarction in the prior 7 days, unstable cardiopulmonary disease or were pregnant.

As part of their work-up for a diagnosis of CAD, patients underwent DSE. Patients were asked to avoid agents that could antagonise the effects of dobutamine (e.g. beta-blockers) for 24 hours prior to their examinations. Following baseline imaging, dobutamine infusion commenced at 10 µg/kg/min and increasing by 10 µg/kg/min at 3 minute intervals, up to 40 µg/kg/min. Termination criteria was the attainment of target heart rate (85 % of maximum age-predicted heart rate), the occurrence of significant symptoms, and the development of wall motion abnormalities. This study was approved by the Oxfordshire Research Ethics Committee (REC No. 08/H0604/127). All participants gave written informed consent.

7.2.2.2. Samples

Citrate blood samples were taken prior to DSE and immediately following DSE. In 9 participants, citrate blood was also taken following administration of contrast agent, prior to DSE, to assess the impact of contrast agent on MP levels. Citrate blood samples were centrifuged at 3000 g for 15 min within 10 min of venepuncture. Plasma was then centrifuged again at 13,000 g for 2 min to produce PPP. 250 μl of PPP was frozen immediately and stored at -80 °C.

7.2.2.3. Effect of Dobutamine on Endothelial Cells *In Vitro*

To determine if dobutamine itself could cause the release of EMPs from endothelial cells, it was used to stimulate HUVECs. HUVECs were cultured in 48-well plates as described in Chapter 3. HUVECS were incubated with 0.05 mg/ml, 0.5 mg/ml and 2.5 mg/ml of dobutamine (Hameln Pharmaceuticals Ltd, Gloucester, UK) in endothelial cell growth media. Supernatant was removed at 1 hour, 5 hours and 25 hours, and analysed for EMPs by flow cytometry, using annexin V, lactadherin, CD31, CD62E, CD106 and CD144 markers.

7.2.3. Measurement of Microparticles

MPs were measured by the optimum flow cytometry assay as described in the Chapter 2. All cell-specific markers described in Chapter 2, were used to stain for MPs.

7.2.4. Statistics

Statistics were performed using GraphPad Prism 5 (GraphPad Software, San Diego). In the Preeclampsia Vascular Study differences in levels between cases and controls were assessed for significance using Mann Whitney tests. In the Dobutamine Stress Echocardiogram Study, changes in MP levels following DSE were assessed by paired non-parametric T-tests (Wilcoxon matched-pairs signed rank tests). Differences in demographics and baseline MP levels between patient groups were compared by Mann Whitney tests. A p-value of <0.05 was considered statistically significant.

7.3. Results

7.3.1. Preeclampsia Vascular Study

7.3.1.1. Subject Characteristics

MP levels were determined in 76 cases with a previous preeclamptic pregnancy and 46 controls with a previous healthy pregnancy. The age, BMI, waist to hip ratio and BP was compared between the two groups to determine any differences in demographics.

Table 7.1 – Demographics of Cases and Controls in Preeclampsia Vascular Study

	Cases (n=76)	Controls (n=46)	p-value
Age	40.5 ± 5.2	40.7 ± 3.2	0.8012
BMI	27.5 ± 6.5	25.1 ± 5.3	0.0604
Waist/Hip Ratio	0.81 ± 0.08	0.80 ± 0.07	0.4443
Systolic BP	119.7 ± 14.3	110.0 ± 9.7	0.0001***
Diastolic BP	78.4 ± 10.2	72.0 ± 7.5	0.0003***

Mean ± SD. P-value calculated from unpaired t-tests.

There was no significant difference between the two groups of participants in their age or waist/hip ratio. However, the cases had significant higher systolic and diastolic BP, compared to controls, although this is still within the normal range of BP. The cases also had a non-significant trend towards a higher BMI (Table 7.1).

7.3.1.2. Microparticle Levels in Cases and Controls

The levels of MPs were compared between the cases and controls, to identify any significant differences in MP levels 5-10 years following either a preeclamptic (cases) or a healthy (controls) pregnancy (Table 7.2).

Table 7.2 – Microparticle Levels in Cases and Controls

MP Subtype	Cases MPs/µl (n=76)	Controls MPs/µl (n=46)	P-Value
Annexin V+ MPs	1254 (900 - 2215)	1637 (1025 - 2625)	0.1236
Lactadherin+ MPs	1379 (959 - 2075)	1545 (1069 - 2619)	0.1236
CD31+CD41+ PMPs	824 (590 - 1553)	958 (475 - 1986)	0.7115
CD31+CD41- EMPs	7.3 (3.9 - 14.0)	8.6 (5.2 - 14.8)	0.3079
CD144+ EMPs	5.2 (2.6 - 9.9)	4.2 (2.3 - 7.6)	0.0988
CD62+ EMPs	5.9 (3.2 - 14.6)	5.0 (2.3 - 14.4)	0.4235
CD106+ EMPs	6.1 (3.6 - 16.0)	8.0 (3.0 - 23.7)	0.4074
CD45+ LMPs	6.8 (3.4 - 13.2)	6.2 (3.5 - 13.0)	0.7795
CD66B+ Granulocyte MPs	4.4 (2.2 - 11.3)	4.4 (1.9 - 13.7)	0.8575
CD14+ Monocyte MPs	5.0 (2.9 - 12.9)	6.5 (2.9 - 10.1)	0.9201
CD3+ T-cell MPs	1.5 (0.6 - 2.7)	1.2 (0.6 - 2.1)	0.2077
Glycophorin A+ RBC MPs	187 (92 - 414)	179 (84 - 557)	0.5701

Median (Inter Quartile Range). P-values calculated by Mann Whitney test

There were no significant differences in any of the MP subtypes between cases and controls.

7.3.2. Dobutamine Stress Echocardiogram Study

7.3.2.1. Subjects

51 patients, 30 males and 21 females, referred for stress echocardiogram testing were eligible and were recruited onto the study.

7.3.2.2. Effect of Dobutamine Stress Echocardiogram on MP levels in all Participants

To examine the effect of DSE on MP levels in individuals with suspected CAD, the effect of DSE testing on MP levels was assessed in all of the participants in the study.

Procoagulant Microparticles

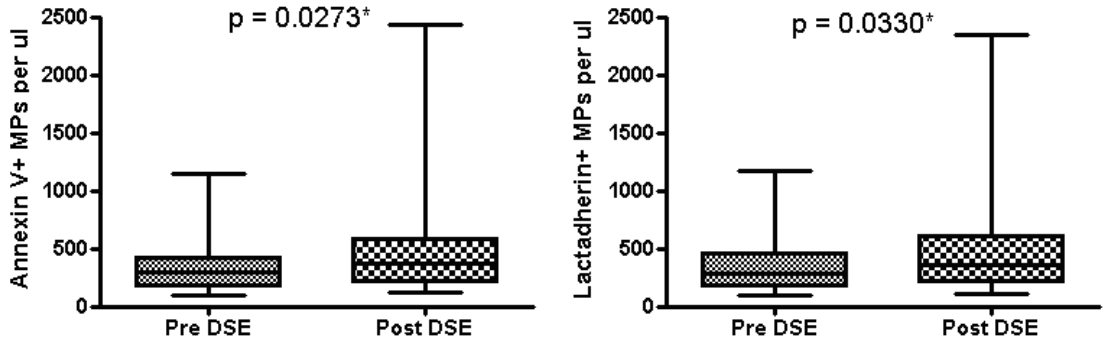

Figure 7.1 – Box and Whisker plot (5-95 percentiles) of annexin V+ and lactadherin+ MPs

Levels of procoagulant MPs, as defined by annexin V and lactadherin binding, were significantly increased immediately following DSE (Figure 7.1).

Platelet-derived Microparticles

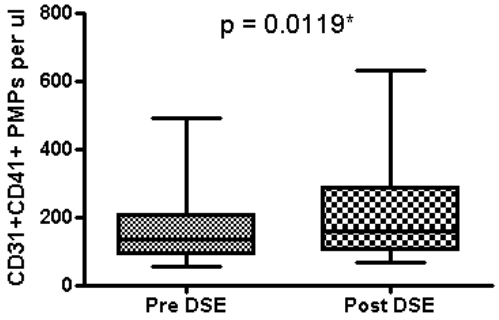

Figure 7.2 – Box and Whisker plot (5-95 percentiles) of CD31+CD41+ PMPs

Levels of PMPs, defined by CD31 and CD41 expression, were significantly increased immediately following DSE (Figure 7.2).

Endothelial Cell-Derived Microparticles

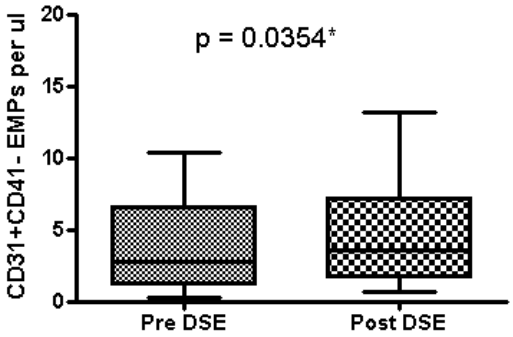

Figure 7.3 – Box and Whisker plot (5-95 percentiles) of CD31+CD41- EMPs

Levels of CD31+CD41- EMPs were significantly increased immediately following DSE (Figure 7.3). Levels of CD144, CD62E and CD106 EMPs were not significantly altered during DSE (data not shown).

RBC-Derived Microparticles

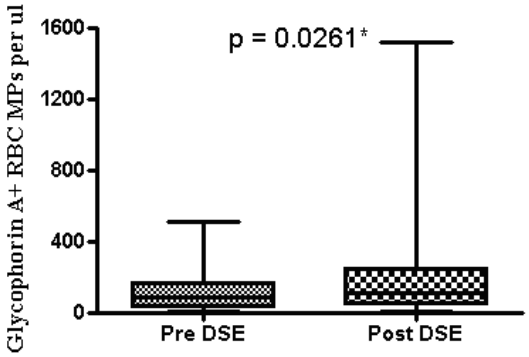

Figure 7.4 – Box and Whisker plot (5-95 percentiles) of Glycophorin A+ RBC MPs

Levels of RBC-Derived MPs, defined by glycophorin A expression, were significantly increased immediately following DSE (Figure 7.4).

Other Microparticle Subtypes

Levels of LMPs, granulocyte-derived MPs, monocyte-derived MPs and T cell-derived MPs were not significantly altered during DSE (data not shown).

7.3.2.3. Effect of Contrast Agent on Microparticle Levels

Contrast agent is used during the DSE to improve imaging of the heart. Therefore, it was important to determine if this was causing the increase in MP levels, rather than the effects of the DSE itself. Levels of MPs were examined before and after infusion of the contrast agent in 9 participants. There was no significant difference in the levels of any MP subtype detected following administration of the contrast agent (data not shown).

7.3.2.4. Microparticle Levels in Participants with a Negative or Positive Dobutamine Stress Echocardiogram

Participants were divided into those with a negative DSE test and those with a positive DSE test, to investigate whether MPs predict the test outcome, and therefore if they may be indicative of atherosclerosis. A positive DSE has a strong positive predictive value for atherosclerosis and CAD (Hennessy *et al.*, 1997).

Table 7.3 – Demographics of DSE Negative and DSE Positive Participants

	Negative DSE	Positive DSE	P-Value
Number	37	14	
Age Mean (SD)	59.6 (12.9)	65.3 (7.3)	0.1568
Males/Females	21/16	9/5	0.6341

P-value calculated from unpaired T-test.

There was no significant difference between the age and gender profile of the two groups. However, there were more participants with a negative DSE than a positive DSE (Table 7.3).

7.3.2.5. Baseline levels of Microparticles in DSE Negative and DSE Positive Participants

To determine if individuals who have a positive DSE result have significantly different baseline levels of MP from individuals with a negative DSE, levels were compared by Mann Whitney Test (Table 7.4).

Table 7.4 – Comparison of baseline MP levels between participants with a negative and positive DSE

	Negative DSE (n= 37)	Positive DSE (n=14)	P-Value
Annexin V+ MPs	300.9 (181.0 - 418.7)	283.4 (119.5 - 765.4)	0.8909
Lactadherin+ MPs	283.6 (192.4 - 463.7)	285.2 (116.9 - 740.8)	0.9747
CD31+CD41+ PMPs	140.9 (89.3 - 196.8)	130.4 (90.2 – 241.4)	0.9916
CD31+CD41- EMPs	3.0 (1.3 – 6.5)	2.2 (0.9 - 8.2)	1.0000
CD144+ EMPs	2.9 (1.2 – 6.8)	3.4 (2.0 - 7.5)	0.3531
CD62E+ EMPs	3.3 (2.1 – 9.1)	5.0 (1.8 – 11.7)	0.6653
CD106+ EMPs	4.1 (2.7 - 10.9)	3.9 (1.6 – 10.4)	0.5904
CD45+ LMPs	7.2 (2.1 - 12.8)	10.5 (3.3 – 19.9)	0.1872
CD66B+ Granulocyte MPs	5.5 (3.0 - 19.8)	5.7 (2.0 – 16.7)	0.8576
CD14+ Monocyte MPs	3.7 (1.6 - 11.7)	7.6 (3.3 – 15.4)	0.2131
CD3+ T-Cell MPs	1.6 (0.9 – 4.5)	1.6 (1.1 - 5.6)	0.6125
Glycophorin A+ RBC MPs	85.9 (30.8 - 164.0)	89.7 (13.1 - 201.4)	0.9579

Median (Inter Quartile Range). P-value calculated by Mann Whitney test

There was no significant difference in the baseline levels of any MP subtype between the participants with a negative or positive DSE. However, CD45+ LMPs and CD14+ monocyte-derived MPs show a non-significant trend to be higher.

7.3.2.6. Change in Microparticles Levels during DSE in DSE Negative and DSE Positive Participants

To determine if individuals with a positive DSE have a different response to DSE, compared to individuals with a negative DSE, the levels of MPs before and after stress echocardiogram were examined.

Procoagulant Microparticles

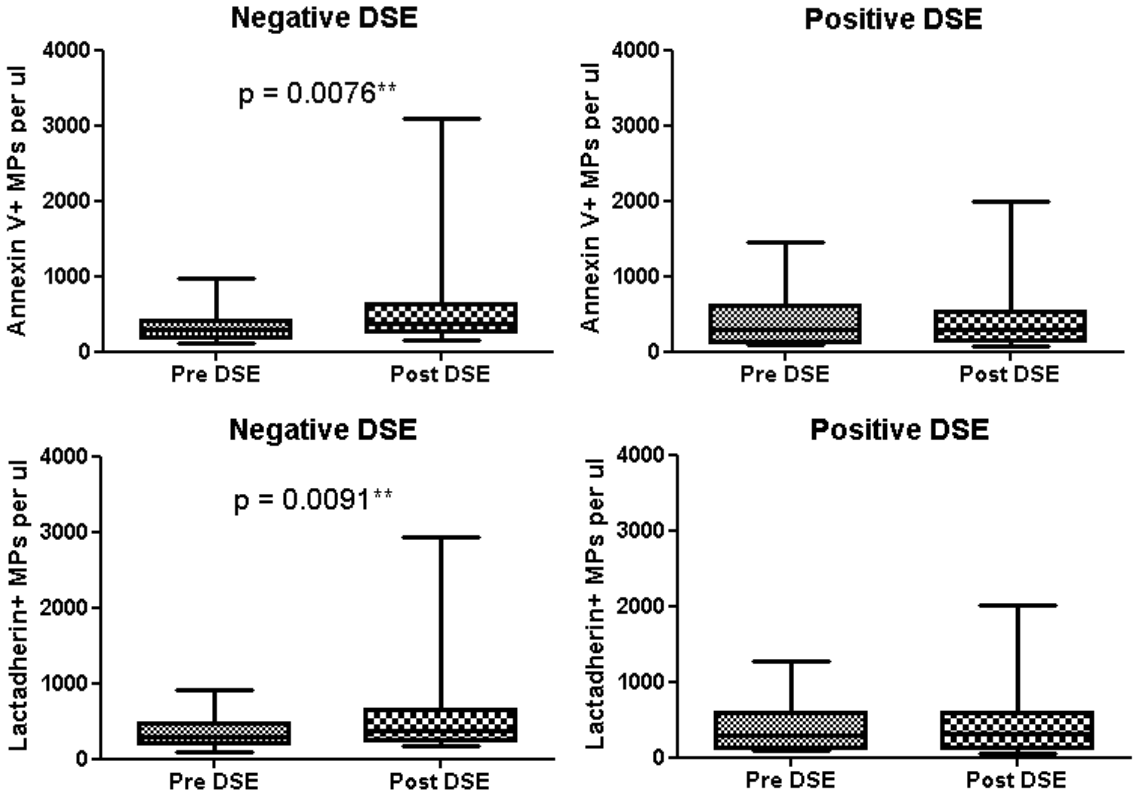

Figure 7.5 – Box and Whisker plot (5-95 percentiles). Procoagulant MPs before and after DSE in individuals with a positive or negative DSE.

The levels of procoagulant MPs were significantly increased immediately following DSE in individuals with a negative DSE (annexin V+ MPs p=0.0076, lactadherin+ MPs p=0.0091), but not in individuals with a positive DSE (Figure 7.5).

Platelet-Derived Microparticles

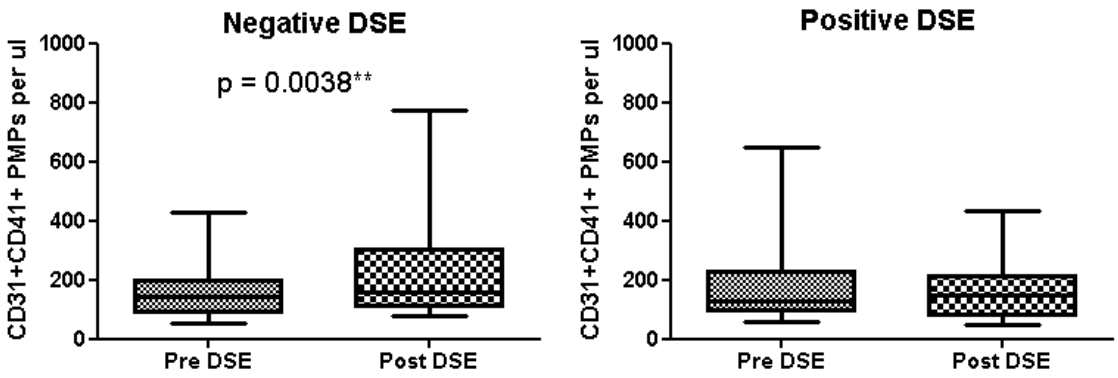

Figure 7.6 – Box and Whisker plot (5-95 percentiles) PMPs before and after DSE in individuals with a positive or negative DSE

The levels of PMPs were significantly increased immediately following DSE in individuals with a negative DSE (p=0.0038), but not in individuals with a positive DSE (Figure 7.6).

Endothelial Cell-derived Microparticles

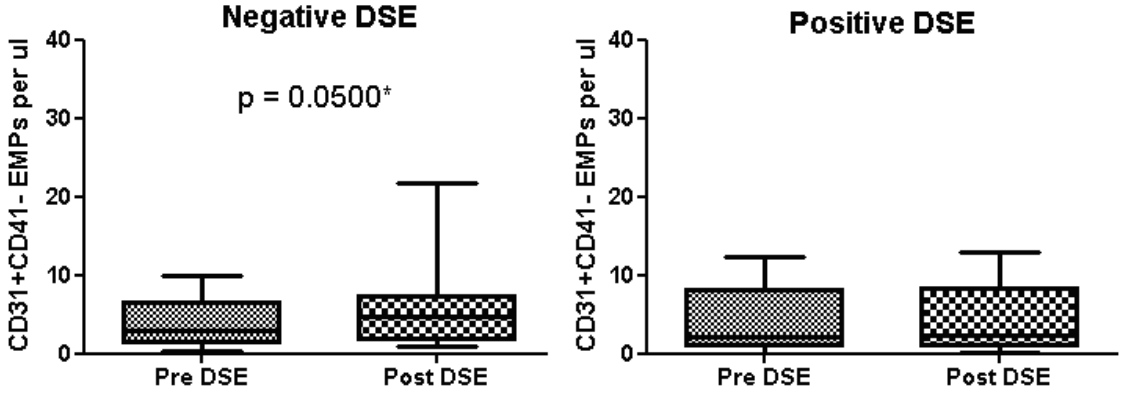

Figure 7.7 Box and Whisker plot (5-95 percentiles) CD31+CD41- EMPs before and after DSE in individuals with a positive or negative DSE

The levels of CD31+CD41- EMPs were significantly increased immediately following DSE in individuals with a negative DSE (p=0.0500), but not in individuals with a positive DSE (Figure 7.7). The levels of CD144+, CD62E+ and CD106+ EMPs were not significantly altered by DSE in either patient group (data not shown).

RBC-Derived Microparticles

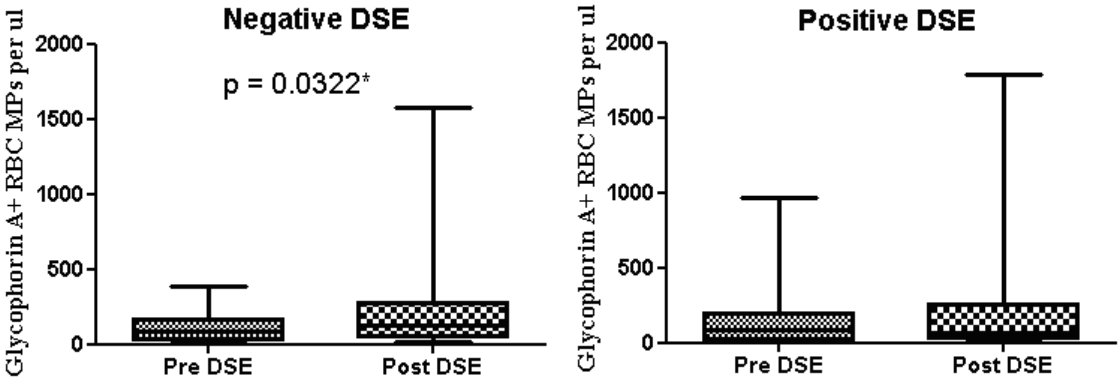

Figure 7.8 – Box and Whisker plot (5-95 percentiles) RBC-derived MPs before and after DSE in individuals with a positive or negative DSE

The levels of RBC-derived MPs were significantly increased immediately following DSE in individuals with a negative DSE (p=0.0322), but not in individuals with a positive DSE (Figure 7.8).

Other Microparticle Subtypes

Levels of LMPs, granulocyte-derived MPs, monocyte-derived MPs and T cell-derived MPs were not significantly altered during DSE in either patient group (data not shown).

7.3.2.7. Effect of Dobutamine on Endothelial Cells *In Vitro*

The incubation of HUVECs with dobutamine did not result in the significant increase in any subtype of EMP at 25 hours post stimulation, compared to incubation with media alone (data not shown). Therefore, dobutamine does not appear to directly stimulate the release of EMPs from endothelial cells *in vitro*.

7.4. Discussion

MPs are known to be elevated in CAD, for example, significantly higher levels of CD31+ EMPs have been reported in CAD patients than in matched controls (Bernal-Mizrachi *et al.*, 2003) and CD144+ EMPs were found to be elevated in type 2 diabetic patients with unstable plaques (Bernard *et al.*, 2009). This raises an interesting question; are MPs elevated in individuals prior to diagnosis of CAD, and therefore would they be a useful prognostic or diagnostic marker?

The two studies in this Chapter examined the levels of MPs in individuals at elevated risk of CVD, to determine if MPs may be useful markers of CVD.

7.4.1. Preeclampsia Vascular Study

Endothelial dysfunction has been demonstrated to be measurable in women 15-25 years following preeclampsia (Ramsay *et al.*, 2003). Therefore, it could be expected that MPs, a marker and a potential cause of endothelial dysfunction, may remain elevated for several years following a preeclamptic pregnancy.

The data in this Chapter indicate that the MP subtypes measured were not significantly different between cases and controls, 5-10 years following the pregnancy. The cases in this study had significantly higher systolic and diastolic BP than the controls, which in itself could have resulted in elevated PMP and EMP levels (Preston *et al.*, 2003), however the mean levels of BP were within the normal range. These results suggest that MPs are not a linking mechanism between preeclampsia and the future increased risk of CVD.

Lok *et al* (2008) measured MP levels six weeks post partum, and found no significant difference between levels of annexin V+ MPs, or percentages of PMPs, EMPs, RBC-derived MPs or placental-derived MPs between preeclamptic patients and normotensive patients. Therefore, perhaps it is not surprising that the current study failed to identify any significant differences, 5-10 years following pregnancy.

7.4.2. Dobutamine Stress Echocardiogram Study

When examining all 51 participants in the Dobutamine Stress Echocardiogram study, a significant increase in procoagulant MPs, PMPs, RBC-derived MPs and CD31+CD41- EMPs following DSE, was observed. These data support the findings of Galloway *et al* (1998), who also found a trend for PMPs to increase after DSE. In this older study, PMPs were measured during DSE, in only 9 patients.

Elevated levels of PMPs have also been identified in healthy individuals, following strenuous exercise (Chaar *et al.*, 2011; Maruyama *et al.*, 2012a). Therefore, inducing

increased heart rate and cardiac stress has previously been shown to increase PMPs in healthy individuals

The finding that procoagulant MPs, PMPs, RBC-derived MPs and CD31+CD41- EMPs were elevated immediately following DSE, suggests that MPs are produced rapidly in response to cardiac stress. There have been few publications on the speed at which MPs are produced and released into the circulation. However, a study has shown that elevated MPs are detectable in the circulation one hour following ingestion of a high fat meal in healthy individuals (Ferreira *et al.*, 2004), suggesting that they can be released very quickly in response to certain stimuli.

The fact that *in vitro* stimulation of HUVECs with dobutamine did not result in a significant increase in any subtype of EMPs even after 25 hours stimulation, suggests that the dobutamine itself is not directly causing the release of CD31+CD41- EMPs from the endothelium. The elevated levels of CD31+CD41- EMPs in patients were found within one hour of administration of dobutamine. Therefore, it seems unlikely that dobutamine itself is triggering the increase in CD31+CD41- EMPs from endothelial cells, and more likely that the increased heart rate and shear stress, induced by the dobutamine, are leading to elevated EMP levels.

Participants were divided into those with a negative DSE and those with a positive DSE, to determine if MP levels predicted the DSE test result, and therefore if they were indicative of atherosclerosis. The participants with a negative DSE make an ideal disease control group, as there are well matched to the DSE positive group and they have very similar CVD risk factors. No significant difference in the baseline levels of MPs was identified, although there was a non-significant trend for LMPs and monocyte-derived MPs to be higher in individuals with a positive DSE.

When the change in MP levels was determined in each patient group, it was found that procoagulant MPs, PMPs, CD31+CD41- EMPs and RBC-derived MPs were all significantly elevated following DSE, in the negative DSE group, but not in the positive DSE group. One hypothesis for this could be that these MPs are released during times of cardiac stress in individuals without atherosclerosis, as a defence mechanism to remove stress and apoptotic signals from the cell. Whereas individuals with atherosclerosis have lost this defensive mechanism, potentially putting them at greater risk during times of cardiac stress. This is supported by the findings that patients with mild stenosis have more circulating CD31+CD42- EMPs than those with severe stenosis (Bernal-Mizrachi *et al.*, 2004).

This study is the first to examine the effect on a wide range of MP subtypes in a large cohort of patients of DSE testing. It benefits from having a well matched group of disease

controls, who have similar demographics and presenting features to the disease group, but who had a negative DSE and are therefore unlikely to have atherosclerosis.

7.4.3. Can Microparticles Predict Future Cardiovascular Events?

Endothelial dysfunction is one of the earliest functional disturbances preceding acute coronary events. Several studies have shown that EMPs are a marker of endothelial activation and damage (Amabile *et al.*, 2005; Wang *et al.*, 2007a). Increased circulating levels of CD62E+ EMPs were found to predict poor outcome in pulmonary hypertension patients (Amabile *et al.*, 2009). Elevated CD31+CD41- EMPs were shown to be an independent predictor of cardiovascular death in end-stage renal failure patients, and were found to have a stronger positive relationship to cardiovascular death than other risk factor, such as history of CVD and Framingham risk score (Amabile *et al.*, 2012).

Elevated plasma levels of CD144+ EMPs may independently predict future cardiovascular events and the addition of CD144+ EMP levels as a parameter to the Framingham risk model significantly improved the predictive power of the model (Nozaki *et al.*, 2009). CD144+ EMPs have also been reported to independently predict future cardiovascular events in patients with heart failure (Nozaki *et al.*, 2010). Another study found that CD144+ EMPs were positively associated with the presence of coronary non-calcified vulnerable plaques in type 2 diabetic patients (Bernard *et al.*, 2009), supporting the findings of Koga *et al* (2005) who noted that elevated CD144+ EMP levels were predictive for the presence of coronary artery lesions, in diabetic patients.

Sinning *et al* (2011) found that annexinV+CD31+ MPs were an independent predictor of cardiovascular events in stable CAD patients. Elevated annexin V+ MPs were also associated with an increased risk of venous thromoboembolism independent of other risk factors (Bucciarelli *et al.*, 2012).

Bulut *et al* (2009) found that annexin V+CD31+ EMPs were elevated in healthy young males with a family history for premature CAD, compared to age, BMI and BP-matched group without a family history. They suggest that the release of EMPs is a very early step in the development of endothelial dysfunction. Therefore, EMPs appear to be a predictive biomarker for future cardiovascular events.

7.4.4. Limitations

Samples obtained from the Preeclampsia Vascular Study were only centrifuged once prior to freezing. However, all samples were processed identically.

The patients from the Dobutamine Stress Echocardiogram Study were divided into those with a negative or positive DSE result. This gives a good indication as to whether these individuals had atherosclerosis or not (Sicari *et al.*, 2008). Ideally patients would have been divided based on their ultimate diagnosis, either with atherosclerosis or without. However, diagnosis is confirmed by angiogram testing, and this data was not available in time for the completion of this thesis.

7.4.6. Conclusions

Levels of MPs were not found to be significantly different in women 5-10 years following a preeclamptic pregnancy, compared to women 5-10 years following a healthy pregnancy. This suggests that MPs may not be the linking mechanism between preeclampsia and future risk of CVD.

DSE significantly increased levels of procoagulant MPs, PMPs, CD31+CD41- EMPs and RBC-derived MPs, suggesting that increased cardiac stress can trigger the release of these MP subtypes. When participants were divided based on the result of their DSE, only those with a negative DSE showed the significant increase in procoagulant MPs, PMPs, CD31+CD41- EMPs and RBC-derived MPs, suggesting that release of these MPs may be a defense mechanism during cardiac stress, and that this defense mechanism is lost in individuals with atherosclerosis.

Chapter 8 – Conclusions and Future Work

8.1. Aims of the Project

The first major aim of this thesis was to develop and validate an assay to measure a wide range of circulating MP subtypes, and to examine the issue of pre-analytical and analytical sources of variability in the measurement of circulating MPs. This was addressed in Chapter 2. Small changes in these variables led to large changes on the levels of MPs detected. This emphasised the importance of methodological standardisation for MP analysis. A standardised flow cytometric assay was chosen for use in this project due to its ability to identify circulating MPs derived from multiple cell types.

The second major aim was to validate the cell specific markers by generating MPs *in vitro* using a variety of agonists, and to determine the impact of agonist used on the phenotype of EMPs released. In Chapter 3, the cell-specific markers chosen were capable of binding to *in vitro* generated MPs. Varying concentrations of physiological agonists were used to generate the release of MPs from cultured endothelial cells. Importantly, the phenotype of the EMPs released was dependent on the stimuli used.

The third major aim was to investigate alternative techniques for MP analysis and examine associations between results from quantitative and functional assays. In Chapter 4, results from three functional MP assays were found to be associated with flow cytometric results for procoagulant MPs and PMPs. These results suggested that the quantitative data on MPs generated from the flow cytometric assay correlated closely with the functional capacity of MPs.

The fourth major aim of this PhD was to measure levels of circulating MPs in healthy individuals to establish a normal range and to analyse variability in levels based on age and gender. In Chapter 5, a healthy normal range was established using 72 healthy individuals. A difference in procoagulant MP and PMP levels between males and females was noted, implying that study designs should gender-match control groups to patient groups, when investigating MPs.

Chapter 5 also aimed to determine the impact of hypoxia on levels of MPs in healthy individuals. Hypoxia, long thought to be a trigger for MP release *in vitro* (Wysoczynski and Ratajczak, 2009), was investigated in a group of healthy individuals *in vivo*. Data in this thesis suggest that exposure to short-term hypoxia can lower procoagulant MP, PMP and RBC MP levels in healthy individuals, indicating an opposing mechanism to the one witnessed *in vitro*.

The fifth major aim was to determine the impact of therapy on levels of circulating MPs and other inflammatory markers in OSA patients and this was examined in Chapter 6. OSA is known to be associated with an increased risk of CVD (Lavie *et al.*, 2000; Peker *et al.*, 2006), and therefore provides a good model for examining patients at a higher risk than the general population. Initiation of treatment for 6 months in minimally symptomatic OSA patients led to a reduction in annexin V+ MPs and an increase in LMPs, possibly indicating a skewing from a procoagulant role to one involving inflammation. Withdrawal of treatment in moderate/severe patients resulted in a significant increase in CD62E+ EMPs, CD106+ EMPs, granulocyte MPs and monocyte MPs. These results provide the first evidence from randomised controlled trials, that MP formation is causally linked to OSA and may promote endothelial activation in these patients.

The final major aim was to examine levels of circulating MPs in cardiovascular-associated diseases. Levels of circulating MPs were analysed 5-10 years following a preeclamptic pregnancy, in Chapter 7. Preeclampsia is a condition which is known to alter circulating levels of procoagulant MPs, LMPs and RBC MPs. These women are also at a long-term higher risk of CVD (Valdiviezo *et al.*, 2012). Results in Chapter 7, suggested that women with a previous preeclamptic pregnancy do not have significantly different levels of circulating MPs compared to women with a previous healthy pregnancy, suggesting that preeclampsia itself may not result in long-term alterations to MPs.

The work described in Chapter 7 also aimed to determine if circulating MP levels provide additional value in the diagnosis of atherosclerosis by DSE. Data in this Chapter showed that procoagulant MPs, PMPs, CD31+CD41- EMPs and RBC-derived MPs were significantly elevated following cardiac stress. When patients were divided based on the results of the DSE, it was found that only patients with a negative DSE have a significant elevation in the above mentioned MPs in response to cardiac stress, whereas patients with a positive DSE, who are likely to have atherosclerosis, appear to have lost this mechanism.

8.2. Microparticles – Cause or Consequence of Pathology?

The question as to whether MPs are effectors in disease or just markers of a pathological process is still much debated. It appears that MPs have the capacity to influence pathological processes, as they can induce thrombosis, inflammation, endothelial dysfunction and angiogenesis. However, they are also released in response to pathological stimuli, suggesting that they can also be used as biomarkers of such processes.

MPs are no longer considered to be just ‘platelet dust’, and it is known that they have many effector functions. They are able to promote and inhibit inflammation, through release of cytokines (Wang *et al.*, 2011) and alteration of endothelial cell adhesion marker

expression (Barry *et al.*, 1998). They also support thrombosis (Bucciarelli *et al.*, 2012), by expressing PS and tissue factor. They can cause endothelial dysyfunction (Brodsky *et al.*, 2004) and they can increase plaque vulnerability through increased angiogenesis (Leroyer *et al.*, 2008a). Therefore, it is unlikely that MPs are innocent bystanders in the development of CVD.

MPs can be generated by apoptosis, however, they can also induce apoptosis in other cells. PMPs from human plasma contain detectable quantities of caspase 3, one of the executioner enzymes of apoptosis, and incubation of PMPs with human macrophages can induce apoptosis in those cells (Boing *et al.*, 2008).

Studies have revealed a direct effect of MPs on endothelial function (Amabile *et al.*, 2005; Brodsky *et al.*, 2004). For example, MPs can induce adhesion of leucocytes to endothelial cells by upregulating adhesion molecules, they can trigger cytokine production (Hong *et al.*, 2012), and they can inhibit NO synthase (Agouni *et al.*, 2008), all suggesting a causal role in pathology. However, annexin V+CD31+ EMP levels are also elevated as a result of endothelial dysfunction (Werner *et al.*, 2006), suggesting that they may be a consequence of pathogenesis.

In vitro studies have led to much of our understanding of MPs derived from leucocytes. Monocyte-derived MP release can be triggered by LPS (Bernimoulin *et al.*, 2009), suggesting that they may be a consequence of disease. However, monocyte-derived MPs have also been shown to cause activation of the endothelial cells, by delivering IL-1β (Wang *et al.*, 2011). The release of granulocyte MPs can be induced by stimulation with TNF-α (Daniel *et al.*, 2006), indicating that they may be produced in response to inflammation. Neutrophil MPs have been shown to increase expression of adhesion molecules on endothelial cells, as well as release of IL-6 and IL-8 (Hong *et al.*, 2012).

Current findings in the literature seem to suggest that circulating MPs are both a cause and a consequence in the pathogenesis of many diseases, and that they have a physiolocial role in health.

8.3. Microparticles – Helpful or Harmful?

MPs derived from several cell types are present in all body fluids under physiological conditions. In fact, the work described in this thesis, showed that MPs can be identified in the circulation of healthy individuals derived from platelets, endothelial cells, monocytes, granulocytes, T cells and RBCs. This suggests that they have a physiological role to play. Elevated levels of MPs have long been considered detrimental to health. However, recent data suggests that MPs may also have beneficial effects in preventing disease (Table 8.1).

Table 8.1 – Physiological and Pathological Roles of Microparticles

MP Subtype	Physiological Role	Pathological Role	References
Procoagulant MPs	Coagulation		(Morel *et al.*, 2006)
	Anti-Coagulant Role in Healthy		(Berckmans *et al.*, 2001)
		Elevated in Thrombosis	(Owens and Mackman, 2011)
		Decreased in Bleeding Disorders	(Nieuwland and Sturk, 2007)
PMPs	Coagulation		(Morel *et al.*, 2006)
	Removal of Harmful Components		(Boing *et al.*, 2008)
	Protective role in Vascular Function in Septic Shock		(Mostefai *et al.*, 2008b)
		Elevated in Thrombosis	(Owens and Mackman, 2011)
		Decreased in Bleeding Disorders	(Nieuwland and Sturk, 2007)
EMPs	Coagulation		(Jy *et al.*, 2005)
	Removal of Harmful Components		(Abid Hussein *et al.*, 2007)
	Stimulation of Vascular Repair		(Dignat-George and Boulanger, 2011)
		Endothelial Dysfunction	(Amabile *et al.*, 2005; Brodsky *et al.*, 2004)
LMPs		Pro-inflammatory	(Mesri and Altieri, 1999)
Granulocyte MPs	Anti-inflammatory		(Dalli *et al.*, 2008)
		Pro-inflammatory in Vasculitis	(Hong *et al.*, 2012)
Monocyte MPs	Coagulation		(Aleman *et al.*, 2011)
		Pro-inflammatory	(Wang *et al.*, 2011)
		Elevated in Thrombosis	(Owens and Mackman, 2011)
T-Cell MPs	Transport of Surface Molecules		(Shefler *et al.*, 2010)
		Endothelial Dysfunction	(Mostefai *et al.*, 2008a)
RBC MPs	Prevents Premature RBC Death		(Tissot *et al.*, 2010)
	Anti-inflammatory		(Sadallah *et al.*, 2008)
		Thrombosis in Sickle Cell Disease	(van Beers *et al.*, 2009)

MPs are an essential component of the coagulation pathway, as demonstrated by the bleeding complications in individuals with Scott Syndrome (Nieuwland and Sturk, 2007). Elevated PMPs are found in autoimmune thrombocytopenia patients who are free of bleeding compared to those with bleeding complications, suggesting a beneficial role in these individuals. However, in the same patients, high levels of PMPs were associated with an increased risk of neurological complications due to cerebral infarcts (Jy *et al.*, 1992), illustrating both the beneficial and harmful role that MPs can play in a single pathological condition. It suggests that MP release is a tightly controlled haemostatic mechanism, which can result in pathology if the control is lost

MPs are also important cell communication vehicles, providing an efficient mechanism of intracellular communication (Diamant *et al.*, 2004; Nomura *et al.*, 2008). MPs can express Fas ligand (FasL) on their surface giving them cytotoxic properties (Jodo *et al.*, 2001). This could potentially result in the apoptosis of harmful cells via FasL binding, giving them a beneficial role, or cause the death of useful cells, resulting in them being classified as harmful (Freyssinet, 2003).

The release of MPs may protect cells by the removal of harmful components. PMPs have been found to contain increased concentrations of caspase 3 (Boing *et al.*, 2008), suggesting that they act as a waste management system for cells (Tushuizen *et al.*, 2011).

EMPs are able to either promote or inhibit inflammation, coagulation and endothelial dysfunction, depending on the physiological context. EMPs may contribute to vascular injury, impair vasorelaxation and promote arterial stiffness (Brodsky *et al.*, 2004) and those expressing PS can also promote thrombosis. However, there are potential beneficial effects of EMPs on endothelial integrity, stimulation of vascular repair, induction of angiogenesis and promotion of endothelial cell survival (Dignat-George and Boulanger, 2011). The inhibition of EMP release from HUVECs causes accumulation of caspase 3 and ultimately cell detachment, suggesting that EMP release is important for endothelial cell survival (Abid Hussein *et al.*, 2007).

In this thesis, CD62E+ EMPs and CD106+ EMPs were found to be elevated during CPAP therapy withdrawal in patients with moderate/severe OSA. CPAP has been shown to improve symptoms and reduce endothelial dysfunction in these patients (Lattimore *et al.*, 2006), so this implies that the rise in EMPs, following CPAP withdrawal, may be detrimental to health.

The finding that patients with mild stenosis have more circulating CD31+CD42- EMPs than those with severe stenosis (Bernal-Mizrachi *et al.*, 2004), suggests that the release of these EMPs in some way protects the endothelial function. This is supported by findings in Chapter 7. The release of procoagulant MPs, PMPs, CD31+CD41- EMPs and RBC MPs

during DSE in individuals with a negative DSE, suggests that release of certain MP subtypes may be a protective mechanism during cardiac stress, and that this has been lost in individuals with a positive DSE, who are likely to have CAD. It may be that the release of CD31+CD41- EMPs is a protective mechanism for the endothelium during times of stress, and that if this mechanism is lost, the viability of endothelial cells deteriorates (Tushuizen *et al.*, 2011). The discrepancy between different subtypes of EMPs may be explained by the nature of their release. CD31+CD41- EMPs are thought to be formed during apoptosis of endothelial cells, whereas elevated CD62E may signify activation of the endothelial cells (Jimenez *et al.*, 2003b).

In Chapter 6, a significant increase in CD45+ LMPs was noted in OSA patients following 6 months of CPAP treatment, which was not seen in patients receiving only supportive care. As CPAP therapy has been shown to reduce symptoms of OSA and endothelial dysfunction (Lattimore *et al.*, 2006), this may suggest that, in these patients, LMPs are actually playing a beneficial role. However, further studies would be needed to determine the role of LMPs in this context.

Acute exposure to hypoxia in healthy individuals led to a significant reduction in procoagulant MPs, PMPs and RBC-derived MPs in Chapter 5. It is possible that circulating levels of these MPs in healthy individuals maintains haemostasis, and that short-term exposure to hypoxia results in a loss of this balance.

It seems that cell-derived MPs can induce both beneficial and detrimental responses in terms of inflammation, coagulation, cell comunication and in the regulation of vascular function. In disease, it may be that the production of MPs is beneficial to the cell releasing them, for the purposes of removing harmful cellular components, but detrimental to the surrounding cells, which are activated in response to these MPs.

8.4. Microparticles Detected by Flow Cytometry – Tip of the Iceberg?

It is thought that the MP population detectable by conventional flow cytometry (above 0.3-0.5 μm in size) (Shantsila *et al.*, 2010) is just the visible 'tip of the iceberg' of all MPs (Lacroix *et al.*, 2010). This has been demonstrated by new technologies, such as atomic force microscopy (AFM), which suggest that flow cytometry underestimates the concentration of vesicles by approximately 1000 fold (Yuana *et al.*, 2010). A study comparing conventional with high-sensitivity flow cytomtery found an 8-20 fold increase in MP counts (Robert *et al.*, 2012). However, many studies conducted that use

conventional flow cytometry show significant differences in MP levels in various clinical settings (Amabile *et al.*, 2009; Ayers *et al.*, 2009; Bernal-Mizrachi *et al.*, 2004).

The patient studies described in this thesis were determined by conventional flow cytometry, as this allows for phenotyping of cell-specific MPs, and it is applicable to routine clinical laboratories. From the literature it was assumed that only larger MPs were detected by this method. However, these studies also identified significant differences in levels between patient groups. Therefore, it appears that the 'tip of the iceberg' may well be proportional to the total population. Future work making use of new techniques, such as AFM, would be able to clarify if the MPs detected by flow cytometry are in fact proportional to the total population.

Another hypothesis is that conventional flow cytometers can actually detect MPs that are much smaller than has been indicated by size-calibrated beads. Van der Pol *et al* suggested that numerous small MPs may be simultaneously illuminated by the laser beam, and are counted as a single event. They named this 'swarm detection'. In polydisperse samples, such as plasma, MPs larger than the detection limit of the flow cytometer would be detected as a single event, whereas those particles smaller than the detection limit would contribute to the MP count by swarm detection. This may explain how flow cytometers are able to detect MPs smaller than 0.3 μm (Van Der Pol *et al.*, 2012).

8.5. Effect of Drugs on Microparticles

The potential pathogenic role of MPs in a variety of disorders makes them therapeutic targets. However, a hurdle in the development of MP-modulating drugs, is the required careful targeting of MPs from specific cell types, in order to gain a beneficial effect, whilst limiting adverse effects (Morel *et al.*, 2011a). Several existing therapies have already been shown to modulate levels of circulating MP. Whether these drugs directly target MPs or whether the observed impact on MPs are due to bystander effects is yet to be demonstrated.

Simvastatin, a statin, was shown to reduce levels of PMPs and monocyte MPs in patients with hypertension and type 2 diabetes (Nomura *et al.*, 2004a; Nomura *et al.*, 2004b). Statins deplete cholesterol from cells, which decreases raft association of proteins and reduces MP shedding (Del Conde *et al.*, 2005), potentially explaining their ability to lower circulating MP levels. Conversely, an *in vitro* study found that Simvastin increased EMP release from HUVECs, and they hypothesised that by inducing EMP release, statins may improve the overall condition of the endothelium (Diamant *et al.*, 2008).

Anti-platelet treatments can also impact on the levels of circulating MPs. Treatment with ticlopidine, an anti-platelet drug, in diabetic patients led to a significant decrease in PMPs and monocyte-derived MPs (Nomura *et al.*, 2004c). Similarly, anti-inflammatory

treatments, including anti-TNF, result in a reduction of circulating annexin V+ MPs, in Crohn's disease patients (Chamouard *et al.*, 2005).

Oxidative stress is a known trigger of MP release, so unsurprisingly treatment of patients following myocardial infarction, with vitamin C (an anti-oxidant) leads to a decrease in PMPs and CD31+ EMPs (Morel *et al.*, 2003). In contrast, postmenopausal women taking hormone replacement therapy were actually found to have higher levels of PMPs, than age-matched controls (Rank *et al.*, 2012), potentially explaining their increased risk of venous thromboembolism.

The findings that commonly prescribed therapeutics can significantly alter MP levels, needs to be considered when designing studies involving MPs. If medications, such as statins, are being used by a patient group, but not in a control group, this could significant skew the results.

8.6. Choice of Endothelial Cell-Derived Microparticle Marker in Cardiovascular Disease

From the studies described in this thesis, it appears that different subtypes of EMPs are produced in response to different stimuli. For example, in the CPAP Withdrawal Study, CD62E+ EMPs and CD106+ EMPs were elevated during therapy withdrawal, whilst CD31+CD41- EMPs and CD144+ EMPs were unaffected. In contrast, in the Dobutamine Stress Echocardiogram Study, CD31+CD41- EMPs were elevated during cardiac stress, whereas CD62E+, CD106+ and CD144+ EMPs were unchanged. Data in Chapter 3 indicated that CD62E+ EMP release was specifically triggered by TNF-α and IL-1α, whereas CD31+ EMPs and CD144+ EMPs release was triggered by multiple stimuli. These findings suggest that there are several different subtypes of EMPs, which are phenotypically distinct, and which are produced in response to different stimuli. This leads to the question of 'which EMP subtype should be measured in CVD?'

EMPs express a wide range of endothelial cell antigens, including those constitutively expressed on endothelial cells, CD31, CD144 and CD146, as well as inducible endothelial markers, CD62E and CD106. Studies investigating EMPs in CVD have used a variety of techniques to define EMP, including CD31+CD41- (Amabile *et al.*, 2012), CD31+CD42- (Wang *et al.*, 2007a), CD144 (Bernard *et al.*, 2009), CD62E (Amabile *et al.*, 2009), CD51 (Bernal-Mizrachi *et al.*, 2003), CD146 (Agouni *et al.*, 2008) and CD105 (Bakouboula *et al.*, 2008). Results in this thesis and in other publications (Jimenez *et al.*, 2003b) suggest that different subtypes of EMPs are released in response to different stimuli. For this reason, it is very difficult to compare and contrast findings from different studies.

CD31 (Platelet-Endothelial Cell Adhesion Molecule) is a commonly used marker for the investigation of EMPs. It is expressed constitutively on endothelial cells and is involved in cell adhesion to the endothelium. It is often used to define EMPs in conjunction with CD41 or CD42, as exclusion markers for PMPs (Chirinos *et al.*, 2005a; Periard *et al.*, 2007; Wang *et al.*, 2007a; Yun *et al.*, 2010). However, even when excluding PMPs, this population can also include LMPs (Amabile and Boulanger, 2011), which are rarely excluded. Although CD31+CD41- was used as an EMP marker in this thesis, it is recognised that the use of negative staining for MP identification is not optimal, and that ideally EMPs should be identified by positive staining.

CD144 (Vascular-Endothelial Cadherin) is an adhesion molecule which mediates homophilic cell adhesion between adjacent endothelial cells, and is believed to be important in vascular permeability. CD62E (E-Selectin) is also an adhesion molecule, which is only expressed on activated endothelial cells. The ligands for CD62E are sialylated carbohydrates expressed on certain leucocytes, making it important in inflammation. CD106 (Vascular Cell Adhesion Protein 1) is an adhesion molecule, which is up-regulated on endothelial cells in response to TNF-α and IL-1. It is the ligand for Very Late Antigen 4 (VLA-4) expressed on leucocytes, and therefore assists in the binding of leucocytes to the endothelium. Whether CD31, CD144, CD62E or CD106 actually play a role in the function of an individual EMP, or if they are just markers of how that EMP was produced is not yet known. However, it is likely that these adhesion molecules may well be important in the contact of EMPs with other cells.

A limitation of this project is that markers were not used to label EMPs in the same tube. This precluded double or triple staining with multiple EMP markers, to determine if CD31+CD41-, CD144+, CD62E+ and CD106+ EMPs were completely separate populations, or if they overlapped. Future work would examine the concurrent labelling of EMPs to establish if certain markers occurred together on the same MPs.

The findings in this thesis suggest that the use of one EMP marker is not sufficient, as EMPs expressing different cell-surface markers are released under different circumstances. CD62E+ EMPs may be a good marker of endothelial activation in individuals at the early stages of CVD, when endothelial damage is not yet apparent. CD31+ and CD144+ may be more appropriate markers in patients where disease has progressed, and apoptosis of the endothelium is a feature. Therefore, when investigating EMPs in CVD it is necessary to use a panel of EMP markers in order to gain as much information as possible about the nature of endothelial injury.

8.7. Future of Microparticle Analysis in Cardiovascular Disease

Circulating MPs are useful markers of cell activation or apoptosis occurring within the body. MPs can be detected in the circulation from cells, which would not normally be easily accessible, such as endothelial cells. The measurement of apoptotic cells is hampered by the fact that cells are rapidly removed by phagocytosis. Therefore, circulating MPs may provide us with an insight into cellular processes that can not otherwise be examined.

Circulating levels of MPs are altered during many pathological processes, they can be associated with disease severity, and they can indicate treatment success. MPs have also been suggested to be potential targets for new therapies. Circulating cell-derived MPs have the capability to be involved in both the promotion and prevention of CVD (Figure 8.1).

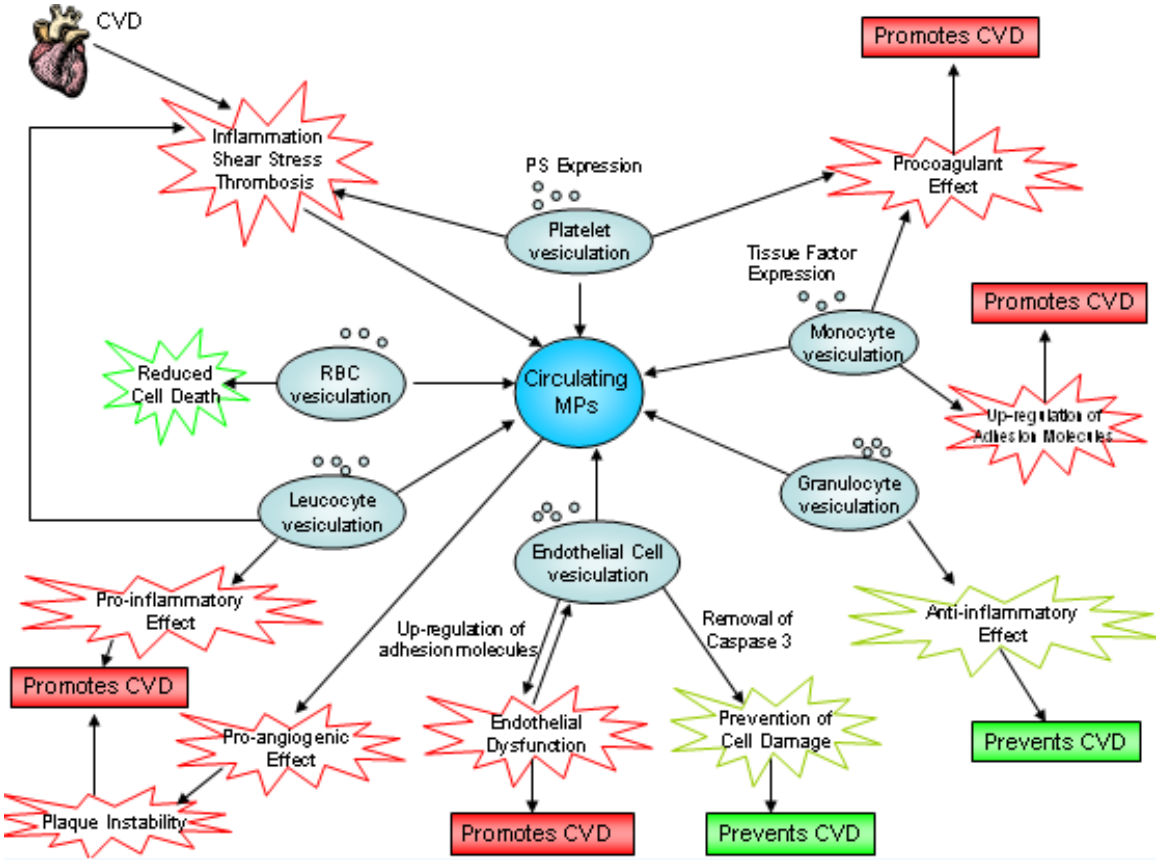

Figure 8.1 – Multiple and Conflicting Roles of Circulating Cell-Derived Microparticles in CVD

Several studies have shown that certain subtypes of MPs are useful in predicting future cardiovascular risk (Amabile *et al.*, 2012; Bulut *et al.*, 2009; Koga *et al.*, 2005; Nozaki *et al.*, 2009; Sinning *et al.*, 2011; Ueba *et al.*, 2010b), as discussed in Chapter 7. If proved conclusively, then there would be benefit in measuring MPs as part of an individuals CVD

risk assessment. The integration of MPs, alongside other biomarkers, could improve the predictive value of risk assessment models (Nozaki *et al.*, 2009).

The use of CVD risk scores can provide a powerful incentive for patients to modify their lifestyle factors, and to reduce their risk (Pell, 2012). Any addition to risk score models that improves their predictive value would be of benefit to those working in risk prevention and their patients.

Data in Chapter 6 on MPs in OSA, suggest that circulating MP levels are altered by CPAP, a therapy that modulates this disease. However, long-term prospective studies would be required in these patients to determine if MPs give prognostic information on future risk of CVD in OSA.

The role of MP analysis may be as an additive marker in the assessment of CAD. Data in this thesis indicate that MPs are released into the circulation in response to cardiac stress, but their release is attenuated in individuals with a higher risk of CAD. The measurement of MPs during DSE could therefore provide additional diagnostic information during the assessment of these patients, although further work would be needed to determine this. Angiogram results from these patients would allow for more definitive stratification of these patients into those with and those without atherosclerosis. Also, the levels of MPs could be assessed periodically over several hours following DSE, to investigate whether patients with a positive DSE result actually have a delayed increase in MP levels.

8.8. What Further Work Should be Done?

As in many studies, the data presented in this thesis raises many questions, several of which I have attempted to address in this Chapter.

This thesis has concentrated on the validation and subsequent use of a flow cytometric assay to quantitatively measure MPs from various cell types. This approach has proved to be very useful in the patient studies, as it revealed that only certain subtypes of MPs were significantly altered. However, this data does not provide functional information about MPs. Chapter 4 revealed that the flow cytometric data on procoagulant MPs was well correlated with assays that measure the functional capability of MPs, suggesting that a quantitative measure is associated with the functional ability of MPs.

In future studies it would be interesting to measure MPs in patient groups by both flow cytometry and by functional assays, to gain more of an understanding into the functional capability of these MPs. In addition to MP markers described in this project, other markers present on MP such as tissue factor, immunoglobulins and complement components, would also be investigated in future experiments, in order to provide additional functional information about the role of MPs in disease.

If MPs are proved to be a useful diagnostic marker or a biomarker for future CVD risk by long-term prospective studies, several issues will need to be addressed. Firstly, samples need to be easily obtainable from patients in clinic, which may well preclude the multiple centrifugation protocol described in this and many other studies (Dey-Hazra *et al.*, 2010; Lacroix *et al.*, 2012). Indeed, in the majority of studies presented in this thesis, it was not possible to obtain plasma that had been centrifuged a second time at 13,000 g. This was due to a lack of equipment and logistical issues within the clinical setting. It is optimistic to assume that all clinics will have the facilities and staff necessary to process samples in this way. Therefore, protocol standardisation needs to take into account the logistics of blood sampling and processing in a clinical setting.

Secondly, a rapid, reproducible and inexpensive standardised assay needs to be available for analysis. The flow cytometric assay described here has been shown to be reproducible in Chapter 2, and the reagents required are relatively inexpensive. However, the assay can be time consuming, and it requires a technician skilled in flow cytometry to perform. The alternative assays described in Chapter 4 offer some advantages in terms of their ability to run multiple samples, however, they do not provide the same level of information regarding the MPs cellular origin.

One major barrier to analysis in clinical samples is the lack of standardisation of pre-analytical variables. The paper resulting from the findings in Chapter 2 (Ayers *et al.*, 2011) made some recommendations for standardisation, along with many other publications (Dey-Hazra *et al.*, 2010; Lacroix *et al.*, 2012; Lacroix *et al.*, 2010; Yuana *et al.*, 2011). However, a single well-described procedure has not been agreed upon, making it difficult to compare results between different laboratories, and ultimately to integrate MPs into routine clinical laboratories.

Finally, circulating levels of MPs would have to provide additional information over existing biomarkers, such as CRP (Koenig *et al.*, 1999) and LDL (Panagiotakos *et al.*, 2003). These existing markers have been well established and have simple sample requirements, as well as reproducible standardised assays. Therefore, as MP analysis requires more complex processing, it will need to give information that can not be obtained by other means.

The mass routine measurement of circulating cell-derived MPs for the assessment of CVD may not yet be ready for introduction. However, with the growing interest in MPs over the past few years, along with a huge increase in recent publications, some of the issues described above may soon be addressed, allowing for a better understanding of MPs and their potential role in CVD.

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Appendix

Appendix 1

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