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The Influence of an Acute Bout of Resistance Exercise on Circulating Endothelial Progenitor Cells and Endothelial Microparticles in Trained Men

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Declaration

Waterford Institute *of* Technology

This thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

The work was done under the guidance of Dr. Michael Harrison at Waterford Institute of Technology.

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Abstract

The endothelium plays an important role in maintaining vascular homeostasis. Endothelial progenitor cells (EPC) are cells which take part in endothelial repair and angiogenesis. These EPCs are typically in low circulating numbers in disease states than healthy populations. Endothelial microparticles (EMP) are blebs of endothelial cell membranes which "bud" off due to endothelial cell activation and apoptosis, and can act as a sensitive biomarker of endothelial status. We hypothesised that low intensity resistance exercise that greatly increased muscle fatigue and acidosis would increase EPCs and endothelial activation.

Aim: To determine the influence of low intensity resistance exercise on EPCs and EMPs.

Methods: Thirteen healthy resistance trained men undertook an acute bout of high volume low intensity resistance exercise (RE). Peripheral blood samples obtained pre- and at 10 min, 2 hr and 24 hr post-exercise. EPCs ($CD34^{+}VEGFR2^{+}CD45^{dim}$) and EMP reflecting endothelial cell activation ($CD62E^{+}$ EMP) and apoptosis ($CD144^{+}$ EMP) were measured by flow cytometry.

Results: Circulating EPCs were increased as a result of the RE bout. Total progenitor cell ($CD34^{+}CD45^{dim}$) counts did not change following exercise however. When EPC counts are expressed as a % of total $CD45^{+}$ events, these were 74% and 110% higher ($p<0.05$) at 2 hr and 24 hr post-exercise respectively. $CD62E^{+}$ EMP but not $CD144^{+}$ EMP increased post-exercise with $CD62E^{+}$ EMP 27% higher at 24 hr post-exercise. These changes in EPC and EMP were accompanied by increases in serum vascular endothelial growth factor (VEGF) and granulocyte-colony stimulating factor (G-CSF). There were no significant changes in other MP subsets.

Conclusions: Low intensity resistance exercise can increase circulating EPCs to promote angiogenesis and vascular repair, possibly via increases in VEGF and G-CSF. Resistance exercise also increases the expression of the activation marker CD62E on endothelial cells which may play a role in binding circulating EPC to the endothelium.

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I would like to thank the following people for their help, guidance and motivation which has allowed me to complete this thesis:

I wish to thank Dr Michael Harrison, my supervisor, for his guidance and teaching (and patience) over the past 2 years. It has been greatly appreciated.

All the subjects who gave up their time to take part in this research, I thank you for the time you gave up to help make my research what it is.

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To Anthony and John, both of whom helped make the research process extremely enjoyable, were always there for support and more importantly comic relief!

To Lisa, Patrick, Chris and Bruno…it's been memorable! The many many tea breaks were of great relief to get away from study and to complain about it instead! Chris, the FIFA rivalry will go on!

Dedication

I wish to dedicate this thesis to Grace, my mum. She has been of great support and a good ear when I needed someone to talk to. She has sacrificed so much for me, I only wish I could one day repay her for everything she has ever done for me.

Mum, it is because of you that I have achieved what I have achieved. Thank you from the bottom of my heart.

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List of Abbreviations

AcLDL- Acetylated Low Density Lipoprotein

AE- Aerobic Exercise

BF- Body Fat

bFGF- Basic Fibroblast Growth Factor

BLa- Blood Lactate

BM- Bone Marrow

CAC- Circulating Angiogenic Cell

CAD- Coronary Arterial Disease

CFU- Colony Forming Unit

CRP- C-Reactive Protein

CXCR4- CXC Chemokine Receptor 4

DM- Diabetes Mellitus

DXA- Dual X-Ray Absorptiometry

EC- Endothelial Cell

EDTA- Ethylene Diamine Tetraacetic Acid

EDV- Endothelial-Dependent Vasodilation

EMP- Endothelial Microparticle

eNOS- Endothelial Nitric Oxide Synthase

EOC- Early Outgrowth Cell

EPC- Endothelial Progenitor Cell

ErMP- Erythrocyte Microparticle

FMD- Flow-Mediated Vasodilation

FS- Forward Scatter

G-CSF- Granulocyte-Colony Stimulating Factor

HGF- Hepatic Growth Factor

HR- Heart Rate

HUVEC- Human Umbilical Vein Endothelial Cell

IAP-1- Inhibitor of Apoptosis Protein 1

IAP-2- Inhibitor of Apoptosis Protein 2

ICAM-1- Intracellular Adhesion Molecule-1

IGF-1- Insulin-Like Growth Factor-1

KDR- Kinase Domain Receptor

KitL- Kit Ligand

LM- Lean Mass

LOC- Late Outgrowth Cell

MAP- Mitogen Activated Protein

$\dot{V}O_2$max- Maximal Aerobic Capacity

miRNA- Micro RNA

MMP- Matrix Metalloproteinase

MP- Microparticle

mRNA- Messenger RNA

NO- Nitric Oxide

PBMNC- Peripheral Blood Mononuclear Cell

PBS-Phosphate Buffered Solution

PDGF- Platelet-Derived Growth Factor

PECAM-1-Platelet/Endothelial Cell Adhesion Molecule

PFP- Platelet-Free Plasma

PGI_2- Prostacyclin

PI3K- Phosphoinositide 3-Kinase

PMP- Platelet Microparticle

PPP- Platelet-Poor Plasma

PS- Phosphatidylserine

RE- Resistance Exercise

RM- Repetition Maximum

ROCK-1- Rho-Associated Coiled-Coil Containing Protein Kinase-1

ROS- Reactive Oxygen Species

SCF- Stem Cell Factor

SDF-1- Stromal-Derived Factor

SS- Side Scatter

TNF- Tumour Necrosis Factor

VCAM-1- Vascular Cell Adhesion Molecule

VE-cadherin- Vascular Endothelial Cadherin

VEGF- Vascular Endothelial Growth Factor

VEGFR2- Vascular Endothelial Growth Factor Receptor 2

$\dot{V}_{O_2}$- Oxygen Uptake

$\dot{V}_{O_2}max$- Maximal Oxygen Uptake

vWf- Von Willebrand Factor

Chapter 1

Introduction

1.0 Introduction

The endothelium plays a crucial role in maintaining vascular health and regulating vascular function. The endothelium secretes a wide range of factors, vasoactive substances (e.g. nitric oxide [NO] and prostacyclin [PGI_2]), pro- and anti-inflammatory and pro- and anti-thrombotic factors. It also plays a role in mechanotransduction (transmission of a mechanical signal to a biochemical response), the adhesion, rolling and transendothelial migration of leukocytes to sites of infection and endothelial cell (EC) damage. However it is difficult to study endothelial biology in humans *in vivo* due to the inaccessibility of ECs.

Endothelial progenitor cells (EPC) are cells which are reside in the bone marrow (BM) (Lyden *et al*., 2001; Devanesan *et al*., 2009) and other storage areas in the body which are recruited to areas of endothelial damage and areas of new blood vessel growth (Asahara *et al*., 1997; Patterson, 2003). They can be mobilised by various factors such as vascular endothelial growth factor (VEGF) (Asahara *et al*., 1999a; Kalka *et al*., 2002a; Kalka et al., 2002b; Heiss *et al*., 2010), stromal-derived factor-1 (SDF-1) (Hattori *et al*., 2001; Yamaguchi *et al*., 2003; Heiss *et al*., 2010) and granulocyte-colony stimulating factor (G-CSF) (Kalka *et al*., 2002b) amongst others. They can directly differentiate into ECs (Hur *et al*., 2004; Yoon *et al*., 2005; Mukai *et al*., 2008) or they can support repair and growth processes through the secretion of growth factors and cytokines (Urbich & Dimmeler, 2004; Urbich *et al*., 2005; Gnecchi *et al*., 2008; Di Santo *et al*., 2009; Wyler von Ballmoos *et al*., 2010). Adhesion molecules such as E-selectin (CD62E), intacellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) bind EPCs to sites of endothelial damage and angiogenesis on

the endothelium (Oh *et al*., 2007). These cells are consistently low in the circulation of those with cardiovascular complications (Fadini *et al*., 2006a; Hill *et al*., 2003) suggesting a decreased ability to mobilise them from the bone marrow, low levels within the bone marrow, or increased senescence of EPCs in the circulation.

Endothelial microparticles (EMP) are small membranous vesicles that are shed from ECs undergoing cell activation and cell apoptosis. These shed vesicles bear proteins present on the EC membrane. This allows MPs to be defined as of endothelial lineage but also provides insights of the cellular processes that lead to EMP release. Increases in EMP bearing the CD31 (Jimenez *et al*., 2003a) and CD144 (Simak *et al*., 2006) antigens likely reflect an increase in EC apoptosis, whereas increases in MPs bearing CD62E (Jimenez *et al*., 2003a), CD54 and CD106 likely reflect an increase in EC activation (Combes *et al*., 1999; Jimenez *et al*., 2003a; Jimenez *et al*., 2005). EMPs may also participate in angiogenesis through aiding basement membrane degradation (Traboletti *et al*., 2002). They have been found to be raised in the circulation of those with cardiovascular disease (Bernal-Mizrachi *et al*., 2004; Werner *et al*., 2006; Lal *et al*., 2009; Sinning *et al*., 2010), and in diabetes (Sabatier *et al*., 2002; Koga *et al*., 2005). EMPs may provide a means of monitoring changes in EC stress as a result of exercise.

There is growing interest in the role of resistance exercise for health. The need for regular resistance exercise is now clearly articulated in current physical activity guidelines (Haskell *et al*., 2007). Resistance exercise has been shown to improve insulin sensitivity in diabetics (Ibañez *et al*., 2005) and improve bone health (Vincent & Braith, 2002). However, resistance training may also contribute to improved cardiovascular health. Resistance training has been shown to improve EC function (Selig *et al*., 2004), improve vessel function as measured by post-occlusion blood flow (Rakobowchuk *et*

al., 2005) and reduce the risk of coronary heart disease risk in men (Tanasescu *et al*., 2002). A high volume, low intensity resistance exercise bout can cause sufficient muscle acidosis indicated by high blood lactate levels and causes high heart rate response (Gotshalk *et al*., 2004). Although the links between aerobic exercise and cardiovascular health are well understood, the mechanisms by which resistance exercise may influence vascular health are under-researched.

Single bouts of aerobic exercise has been shown to improve circulating EPC numbers in some (Adams *et al*., 2004; Rehman *et al*., 2004; Laufs *et al*., 2005; Van Craenenbroeck *et al*., 2008; Goussetis *et al*., 2009; Möbius-Winkler *et al*., 2009; Thorell *et al*., 2009; Bonsignore *et al*., 2010; Sandri *et al*., 2011; Van Craenenbroeck *et al*., 2011) but not all studies (Shaffer *et al*., 2006; Van Craenenbroeck *et al*., 2010). These increases in EPCs are sometimes accompanied by increases in VEGF (Adams *et al*., 2004; Sandri *et al*., 2011). A limited number of studies have also investigated the effects of acute aerobic exercise on EMP and MP numbers, with studies showing no change in EMP levels (Sossdorf *et al*., 2010; Chaar *et al*., 2011; Sossdorf *et al*., 2011). However, the same authors found significant increases in platelet MP (PMP) levels (Sossdorf *et al*., 2010; Sossdorf *et al*., 2011; Chaar *et al*., 2011). Increases in EMP levels are likely to reflect EC stresses by which a healthy adaptation can occur. To date no studies have investigated the influence of resistance exercise on circulating EPC and EMP levels.

The primary purpose of this study was to examine the influence of a high volume low intensity resistance exercise bout on circulating EPC levels in young resistance trained men. Changes in EPC reflect the capacity for endothelial repair and angiogenesis whereas changes in $CD62E^{+}$ EMP reflect the ability of the endothelium to bind circulating blood cells including EPCs. Cytokines and growth factors known to mediate

EPC mobilisation and homing were also measured in order to explain potential changes in EPC. EMPs bearing EC activation and apoptotic markers were enumerated in order to examine the cause of EMP release. A secondary purpose of the research was to examine the influence of aerobic exercise on EMP levels in peripheral blood in aerobically trained young men.

Chapter 2

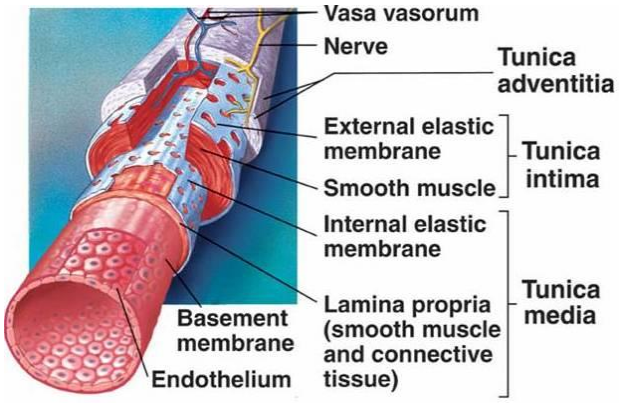
Literature Review

2.0 The Literature Review

2.1 The Endothelium

The endothelium, which consists of a one cell thick layer of endothelial cells (EC), describes the inner layer of blood vessels, whether they are arteries, arterioles, capillaries, veins or venules (figure 2.1). The endothelium is a semi-permeable barrier, which separates components of the blood from the surrounding tissue (Toya & Malik, 2011).

Figure 2.1. Diagram of the Different Layers of Blood Vessels



Seeley et al., 2010

The endothelium was first described by His (1865). He stated that the endothelium was different than the epithelia, as the endothelial layer of blood vessels could allow the passive movement of serum through its layers, whereas the epithelial tissue could not. However, he wrongly stated: “we have no reason to ascribe to endothelia any secretory functions” (His, 1865, pg.18).

In fact the endothelium has many secretory functions, one being secreting vasoactive substances which control blood vessel diameter, such as nitric oxide (NO) and prostacyclin (PGI_2). ECs also secrete many growth factors, vasodilatory, vasoconstrictive, pro- and anti-thrombotic as well as pro- and ant-inflammatory factors.

The EC membrane expresses receptors for vascular endothelial growth factor (VEGF), such as VEGF receptor 2 (VEGFR2), otherwise known as kinase domain receptor (KDR), which plays a part in EC tube formation, migration and proliferation (Mukhopadhyay *et al*., 2007). The EC membrane consists of proteins involved in inflammation such as intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) (Friedman *et al*., 1986; Korenaga *et al*., 1997) as well as E-selectin (CD62E) (Kansas, 1996). These adhesion molecules also play a role in the adhesion and transendothelial migration of inflammatory cells (Jia *et al*., 1999).

ECs also have a role in mechanotransduction, whereby mechanical forces such as those related to blood flow and blood pressure are transmitted intracellularly to induce a biochemical response. This is very important as vasoactive substances can be released in response to blood flow across the EC.

The EC layer also functions to act as a barrier to regulate the movement of solutes and fluid from the circulation into the tissues. VE-cadherin is a protein which plays an important part in regulation of these solutes and fluids by maintaining tight junctions between ECs (Iyer *et al*., 2004).

2.2 Endothelial-Dependent Vasodilation

Endothelial-dependent vasodilation (EDV) encompasses the endothelium's role in mediating vasodilation of the blood vessel through its ability to secrete NO and PGI_2. Furchgott & Zawadzki (1980a, 1980b) first discovered this role of the vascular endothelium in controlling blood flow. These substances exert their effects on the vascular smooth muscle resulting in a relaxation effect, thus a vasodilation of the vessel in question occurs. Interestingly, prostaglandins contribute more to vasodilation of blood vessels in skeletal muscle rather than the well known NO (Koller & Kaley, 1990). EDV is typically measured by flow-mediated dilation (FMD) (Bernal-Mizrachi *et al*., 2004).

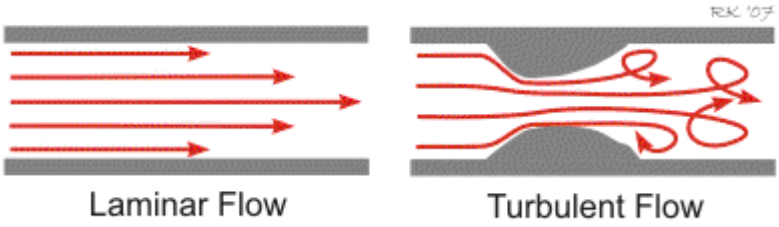
Endothelial dysfunction is suggested to be due to a loss of ECs and reduced production of NO and other vasoactive substances from within ECs. Endothelial dysfunction occurs in a number of pathologies, including coronary arterial disease (CAD) (Bernal-Mizrachi *et al*., 2004) and diabetes mellitus (DM) (Nappo *et al*., 2002). Smoking, obesity, dyslipidemia, hypertension, and hyperglycemia are critical factors that adversely affect endothelial function, whereas exercise training enhances endothelial function (Clarkson *et al*., 1999).

2.3 Endothelial Mechanotransduction

Mechanotransduction is the process by which a mechanical signal is transmitted into a biochemical signal. The mechanical signals which we refer to are shear stress (effect of blood flow on the endothelium) and cyclic strain or stretch (an effect of blood pressure). Blood flow can be laminar or and turbulent. Both forms of flow generate shear stress

(dragging effect of blood against ECs) on the EC wall, and tensional stretch on ECs. However, these two forms of flow have very different effects on their surrounding environment.

Figure 2.2. Forms of Blood Flow.



Retrieved from http://www.cvphysiology.com/Hemodynamics/H007.htm. Accessed 19th July 2011

Laminar shear causes ECs to be spindle shaped and aligned in the direction of flow, whereas ECs in areas of turbulent flow are rounded in shape, with no uniform alignment (Langille & Adamson, 1981). In these areas of flow, EC apoptosis is increased and there is an increase in size of leakage junctions allowing invasion of the EC layer by leukocytes and plasma, contributing to atheroma (Berardi & Tarbell, 2009). This is supported by the observation that the most common sites of atherosclerotic plaque development appear to be at arterial branching sites, where there is turbulent flow (Bai *et al*., 2010).

ECs that line the blood vessels express receptors such as the seven-span-receptor-coupled G protein (Gudi *et al*., 1996), Smad6, and Smad7 (Topper *et al*., 1997) all of which detect mechanical forces. These receptors, when activated promote cell signalling pathways to respond to the stimuli, e.g. one minute after the onset of shear stress (blood flow across the endothelium) there is an increase in cytoplasmic calcium ion (Ca^{2+})

concentration in ECs (Yamamoto *et al*., 2000). The membrane-attached receptors promote cell signalling pathways by interacting with cytoskeletal proteins, transmembrane enzymes, integrins, ion channels, caveolae, G-protein receptors and tyrosine kinase receptors (Lehoux *et al*., 2006).

There has been a recent interest in a structure called glycocalyx. This structure provides an extracellular sensing system that transduces mechanical shear stress to intracellular biochemical signals (figure 2.3). The glycocalyx is a carbohydrate-rich layer anchored to the endothelial membrane, and it has roles in regulating vascular permeability (van Haaren *et al*., 2003), and buffering the interaction between the EC layer and erythrocytes (Vink *et al*., 2000). The glycocalyx consists of a protein core, glycoproteins, proteoglycans, glycosaminoglycans and associated plasma proteins. The glycoprotein backbone consists of core proteins such as syndecans and glypicans. The proteoglycan components are connected to the EC membrane by the syndecans, which have membrane spanning domains (Fransson *et al*., 2004). The glycocalyx is ~0.5μm thick in capillaries located in the muscle (Vink & Duking, 1996), 2-3μm in small arteries (van Haaren *et al*., 2003), and up to 4.5μm in carotid arteries (Megens *et al*., 2007). It is suggested that fluid shear stress exerted by the blood is 'sensed' by the core proteins of the glycocalyx, and this mechanical force is transmitted to the intracellular actin cytoskeleton via the glycocalyx transmembrane section (Yao *et al*., 2007). Syndecans are hypothesised to be the major molecules involved in this process, by inducing the displacement of actin filaments thus initiating downstream intracellular signalling cascades (Thi *et al*., 2004). In fact, the glycocalyx, being associated with the cell cytoskeleton, can mediate NO release via the association of the cytoksleton and platelet/EC adhesiom molecule-1 (PECAM-1) (Dusserre *et al*., 2004), whereby

mechanical disruption of the glycocalyx can lead to activation of the PECAM-1 molecule. In disease states the glycocalyx layer is reduced in thickness compared with healthy controls (Nieuwdorp *et al*., 2006; van den Berg *et al*., 2006) indicating the impaired ability of those with disease states to manufacture and release NO as a result of shear stress.

2.4 Shear Stress-Mediated Cell Signalling

Insights into the influence of different patterns of shear stress on intracellular signalling have been gained from cell culture models in which ECs are plated in viscometer discs and subjected to fluid shear stress. Shear stress is involved in intracellular Ca^{2+} release (Yamamoto *et al*., 2000; Oancea *et al*., 2006) and EC protection (Taba *et al*., 2003).

Shear stress can cause ECs to be increasingly resistant to apoptosis. In fact shear stress can also initiate the mitogen-activated protein (MAP) kinase cascade, which upregulates protein synthesis to strengthen the cytoskeleton (Lehoux & Tedgui, 1998). This may increase cell resistance to apoptosis, as one step in apoptosis is the degradation of the actin cytoskeleton (DeMeester *et al*., 1998). Shear stress can also prevent cell apoptosis through the increase in inhibitors of apoptosis proteins 1 and 2 (IAP1 and IAP2) (Taba *et al*., 2003). These proteins lead to the breakdown of caspases; molecules involved in cell apoptosis, particularly caspase 3 (LeBlanc, 2003). The breakdown of caspases occurs through the direct interaction of IAPs and caspases, or via ubiquitination or proteasomal degradation. Pi *et al* (2004) observed that shear stress inhibited EC apoptosis, but shear stress has also been shown to stimulate cell growth, with further support for shear stress increasing the number of apoptosis resistant proliferative ECs after initial apoptosis caused by VEGF deprivation (Sakao *et al*., 2005). Shear stress

may also induce the phosphoinositide 3-kinase (PI3K)-mediated phosphorylation of Akt in a time-dependent manner (Dimmeler *et al*., 1998), and Akt has been shown to phosphorylate the protein Bcl-associated death protein, which is a proapoptotic protein (Peso *et al*., 1997). When phosphorylated, Bad's activity is inhibited. The shear stress-mediated inhibition of EC apoptosis may be one mechanism by which the vascular integrity could be maintained. On the other hand, one study found that shear stress stimulates the increased production of CYP1A1 an enzyme produced through activation of aryl hydrocarbon receptor (Han *et al*., 2008). This enzyme is said to be involved in cell cycle arrest induced by shear stress. However, the overwhelming evidence to date suggests that shear stress prevents EC apoptosis.

Shear stress also causes NO release from ECs. Shear stress stimulates the increased expression of endothelial NO synthase (eNOS) messenger RNA (mRNA) (Tao *et al*., 2006), via Akt phosphorylation mediated by PI3K (Fisslthaler *et al*., 2000; Zhang *et al*., 2006), and AMPK-mediated phosphorylation of eNOS (Zhang *et al*., 2006), indicating improved NO production by ECs. NO may exert a vasoprotective effect by inhibiting platelet aggregation (Benjamin *et al*., 1991), leukocyte adhesion (Kubes *et al*., 1991) and smooth muscle cell proliferation (Scott-Burden & Vanhoutte, 1993).

Figure 2.3. Pathways by which Shear Stress Mediates its Effects Intracellualrly on Endothelial Cells.

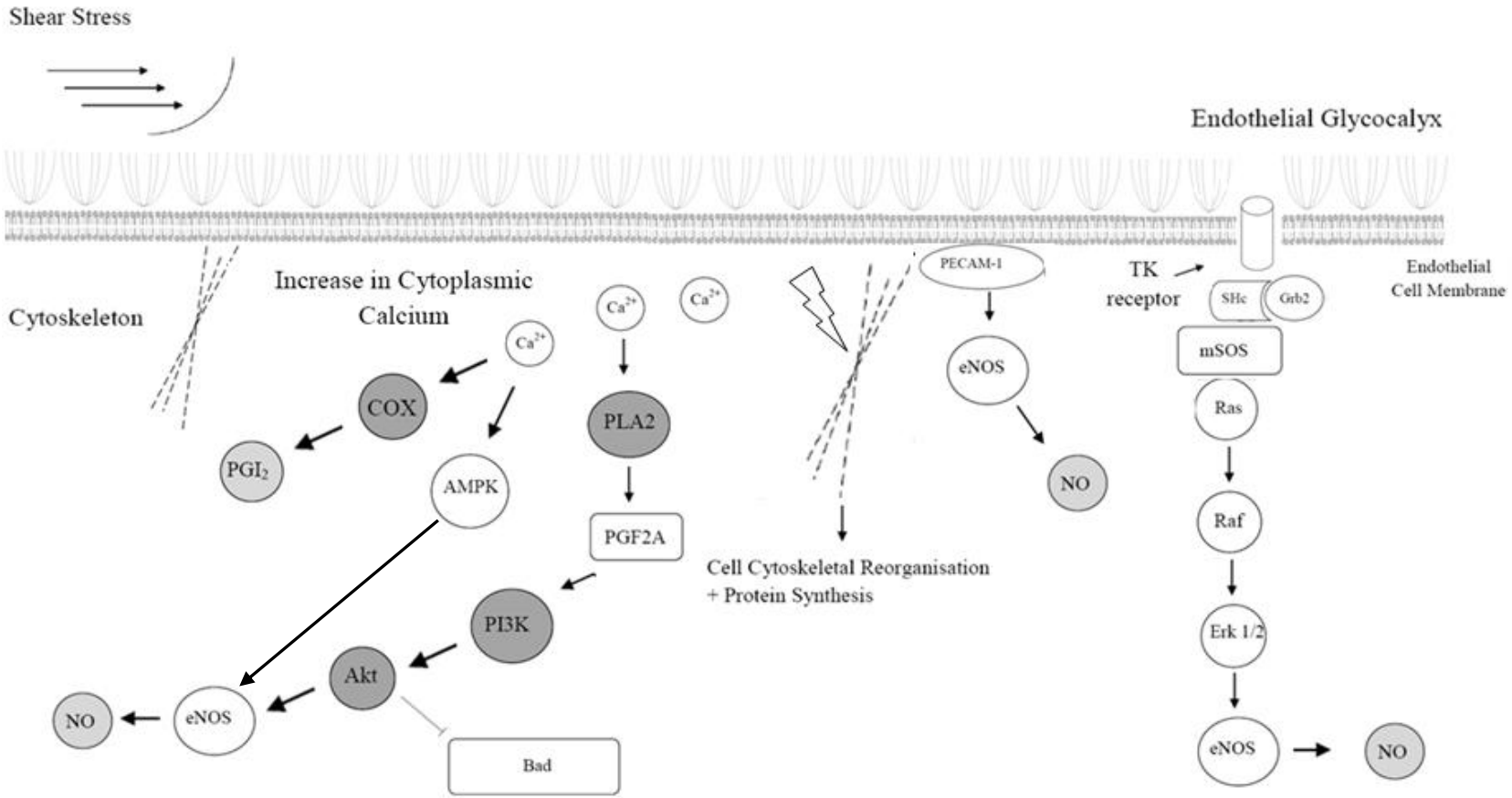


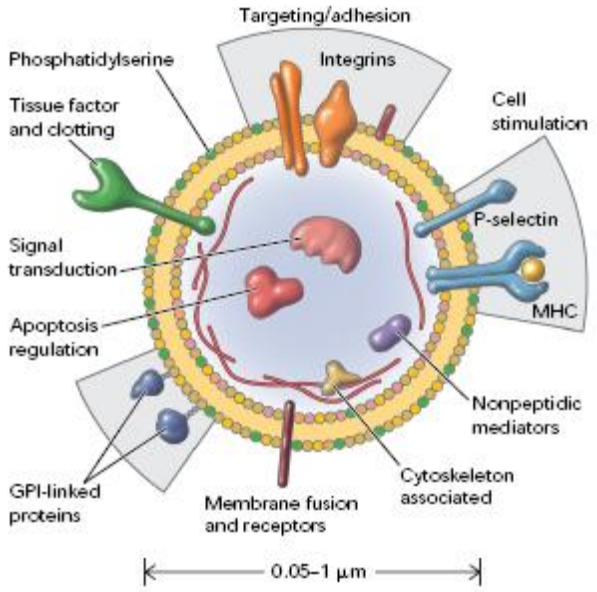
Figure illustrating the process by which shear stress is transduced into a biochemical response. Increase in cytoplasmic calcium (Ca^{2+}) causes release of Nitric Oxide (NO) through activation of Phospholipase A2 (PLA2) and 5' AMP-activated protein kinase (AMPK).Ca^{2+} also stimulates the production of Prostacyclin (PGI_2) via Cyclooxygenase (COX) activation. Shear stress also stimulates the production of NO through mechanical stress at the glycocalyx, which is linked to the cell cytoskeleton, and through activation of tyrosine kinase (TK) receptor. PGF2A=Prostaglandin PGF2A, PI3K= Phosphoinisitide 3-kinase, eNOS= endothelial NO synthase, Bad = Bcl-associated death protein, PECAM-1 = Platelet/ endothelial cel adhesion molecule 1, Ras = RAt sarcoma, Raf =Ras-associated factor, ERK1/2 = Extracellular signal-regulated kinases 1/2. Adapted from Weinbaum et al., 2003; Ando & Yamamoto, 2009

2.5 Endothelial Microparticles

2.5.1 Overview

Microparticles (MP) are small (<1µm) cell-derived membranous 'blebs' which are released from cells under conditions of activation or apoptosis. They have been shown to be released from ECs (endothelial microparticles; EMP), platelets (platelet microparticles; PMP), vascular smooth muscle cells, leukocytes (leukocyte microparticles; LMP) and erythrocytes (erythrocyte microparticles; ErMP) (VanWijk *et al*., 2003).

Figure 2.4. Cellular-Derived Microparticle.



Hugel et al., 2005

PMPs are the most numerous MPs in peripheral human circulation (George *et al*., 1982; Hunter *et al*., 2008), making up to 70-90% of all MPs (Horstman & Ahn, 1999). This

section will focus primarily on EMPs, with sections describing the composition, function and detection of EMPs, as well as the effects of exercise on MP number.

2.5.2 Endothelial Microparticle Composition

MP membranes consists of a phospholipid bilayer with many proteins embedded. The proteins they carry are identical to that of its parent cell where it was shed from, i.e. MPs derived from ECs will express endothelial markers. EMPs express the markers CD31 (PECAM-1), CD51 (integrin α_v), CD54 (ICAM-1), CD105 (endoglin), CD106 (VCAM-1), CD146 (melanoma cell adhesion molecule), CD62E (E-selectin) and CD144 (VE-cadherin) (Shet *et al*., 2008; Chironi *et al*., 2009; Sossdorf *et al*., 2011).

MP membranes are also enriched with negatively charged phospholipids (Combes *et al*., 1999). In the rested unactivated state, negatively charged phospholipids are only present on the inner leaflet of the cell membrane bilayer. During MP shedding negatively charged phospholipids transolcate from the inner part of the membrane to the outer membrane (VanWijk *et al*., 2003; Diamant *et al*., 2004; Boulanger *et al*., 2006). The phospholipid content of MPs membranes is shown in table 2.1.

Table 2.1. Phospholipid Content of Microparticle Membranes.

Phospholipid	**%**
Phosphatidylcholine	59
Sphingomyelin	20.6
Phosphatidylethanolamine	9.4
Phosphatidylserine	1.05
Other	<10

Weerheim et al., 2002

Phosphatidylserine (PS) is the principal negatively charged phospholipid of this group, and it is this negative charge which contributes functionally to the procoagulant activity of MPs, as the tenase and prothrombinase complexes (which are involved in coagulation and the formation of thrombin) assemble on PS (Sinauridze *et al*., 2007). Consequently, PS is particularly relevant to MP function when studying various thrombotic disorders.

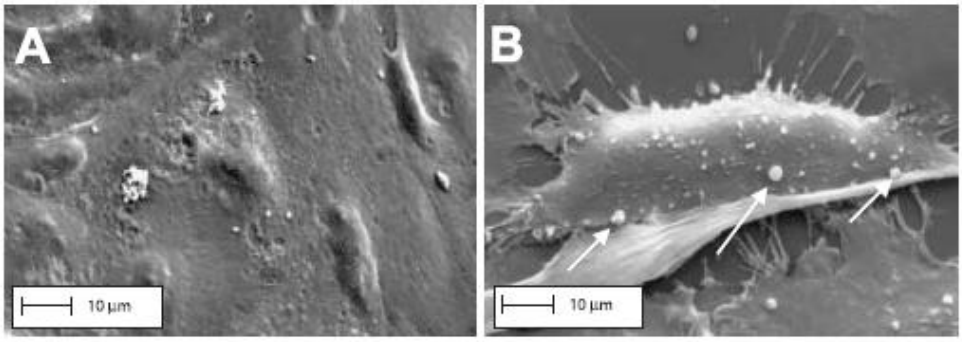
EMPs also express matrix metalloproteinases (MMP) (Taraboletti *et al*., 2002; Lozito & Tuan, 2011) such as MMP-2 and MMP-9. These enzymes play a role in basement cell matrix degradation (Taraboletti *et al*., 2002), a process required for angiogenesis (new blood vessel growth).

The MP core contains genetic material, such as messenger RNA (mRNA) (Deregibus *et al*., 2007; Ullal *et al*., 2010) and micro RNA (miRNA) (Hunter *et al*., 2008). One theory of MP function is that they serve as vectors of this material facilitating its targeted and packaged delivery to distant cells (Hunter *et al*., 2008).

2.5.3 Formation

MPs are formed by cell activation and cell apoptosis (Jimenez *et al*., 2003a). Cell activation can be caused by increased shear stress and high circulating levels of thrombin, tumour necrosis factor (TNF)-α (Jimenez *et al*., 2005) and IL-6 (Ueba *et al*., 2010). It can also be caused by high levels of intracellular Ca^{2+} and ADP (Jimenez *et al*., 2005). Cell apoptosis can be caused by hypoxia, hyperoxia, high blood glucose and periods of growth factor deprivation (Hogg *et al*., 1999; Min *et al*., 1999).

Figure 2.5. Scanning Electron Microscope Images of Unstimulated Human Umbilical Vein Endothelial Cells (HUVECs) (A) and Stimulated Cells showing the Formation of Microparticles (white arrows) (B).



Diamant et al., 2004

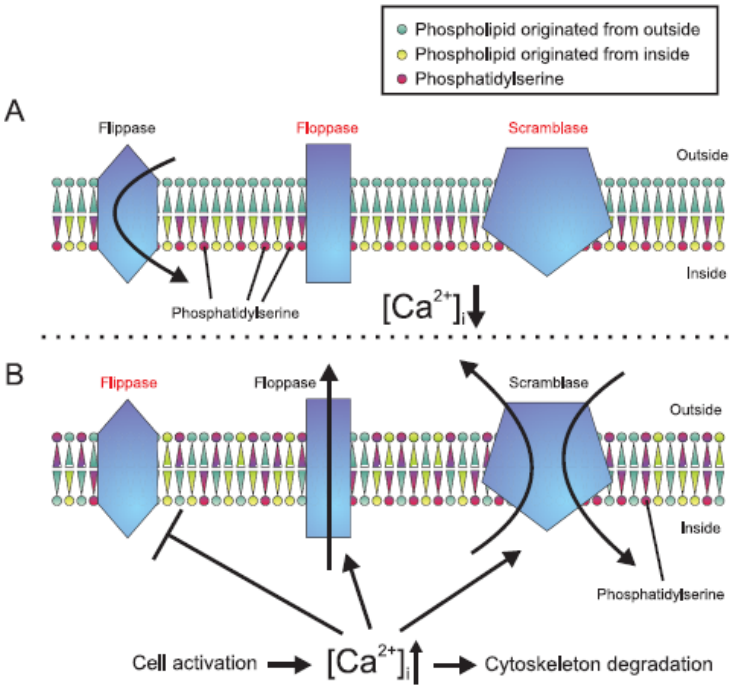
Cytoskeleton disruption appears to be necessary for MP formation (Yano *et al*., 1994). Caspases (a family of proteinases) and Rho-associated coiled-coil containing protein kinase-1 (ROCK-1) appear to be involved when MP formation is triggered by both cell activation (Sapet *et al*., 2006) and cell apoptosis (VanWijk *et al*., 2003). The mechanism of MP formation by apoptosis involves cell contraction, DNA fragmentation, disruption of the cell cytoskeleton caused by increased ROCK-1 activity and dynamic membrane blebbing (VanWijk *et al*., 2003). ROCK-1 also appears to play an important part in the contraction of the actin-myosin network within the cell ultimately leading to apoptosis (Sebbagh *et al*., 2001; Jimenez *et al*., 2005).

Caspase 3, is believed to play an important role in apoptotic MP formation through enucleation; the removal of the cell nucleus (Ahn, 2005). Caspase 3 removal minimises or prevents cell apoptosis, thus confirming its role in this process. However, in PMP formation, calpain rather than caspase 3 led to apoptotic changes in platelets which led

to membrane blebbing (Wolf *et al*., 1999). This is not surprising since platelets do not contain a nucleus. Calpain does this through hydrolysing actin binding proteins and thus decreases actin association with cell membrane glycoproteins (Fox *et al*., 1991). However, several groups indicate that calpain action may not be absolutely necessary for MP formation (Wiedmer *et al*., 1990; Fox *et al*., 1991). The evidence to date suggests that either caspase 3 or calpain plays an important role in MP formation, with caspase 3 involved in nucleated cell MP formation, and calpain involved in enucleated cell MP formation.

Several research groups have indicated that an increase in intracellular Ca^{2+} is required for MP formation (Coleman *et al*., 2001; Sebbagh *et al*., 2001). Cellular activation can cause this influx of Ca^{2+} from the endoplasmic reticulum, and this increase in intracellular Ca^{2+} may directly lead to activation of scramblase and floppase, leading to a downstream inhibition of flippase (Hugel *et al*., 2005). These enzymes stimulate a loss of membrane lipid asymmetry (Daleke *et al*., 2003). Thus the role of Ca^{2+} is important in MP formation; it induces cell proteolysis potentially leading to alterations in membrane phospholipid composition (Freyssinet & Toti, 2010). This loss of membrane lipid asymmetry involves the translocation of negatively charged phospholipids from the inner membrane to the outer membrane contributing to MP formation (VanWijk *et al*., 2003; Diamant *et al*., 2004; Boulanger *et al*., 2006). These lipid molecules also have an important role in the amplification of thrombin generation (Simak & Gelderman. 2006). As a result of membrane phospholipid translocation, covalent links that hold the cell cytoskeleton and the membrane together are disturbed, thus promoting MP formation (Montoro-García *et al*., 2011).

Figure 2.6. Regulation of the Asymmetric Phospholipid Distribution on Cell Membranes by Scramblases, Floppases and Flippases.



A= Resting cell conditions. Low cytoplasmic calcium concentrations and active flippase

B= High cytoplasmic calcium concentrations cause inhibition of flippase, and a concomitant activation of floppase and scramblase initiates the loss of phospholipid asymmetry and exposure of phosphatidylserine on the outer membrane

Burnier et al., 2009

Figure 2.7. Pathways for Cellular Microparticle Release.

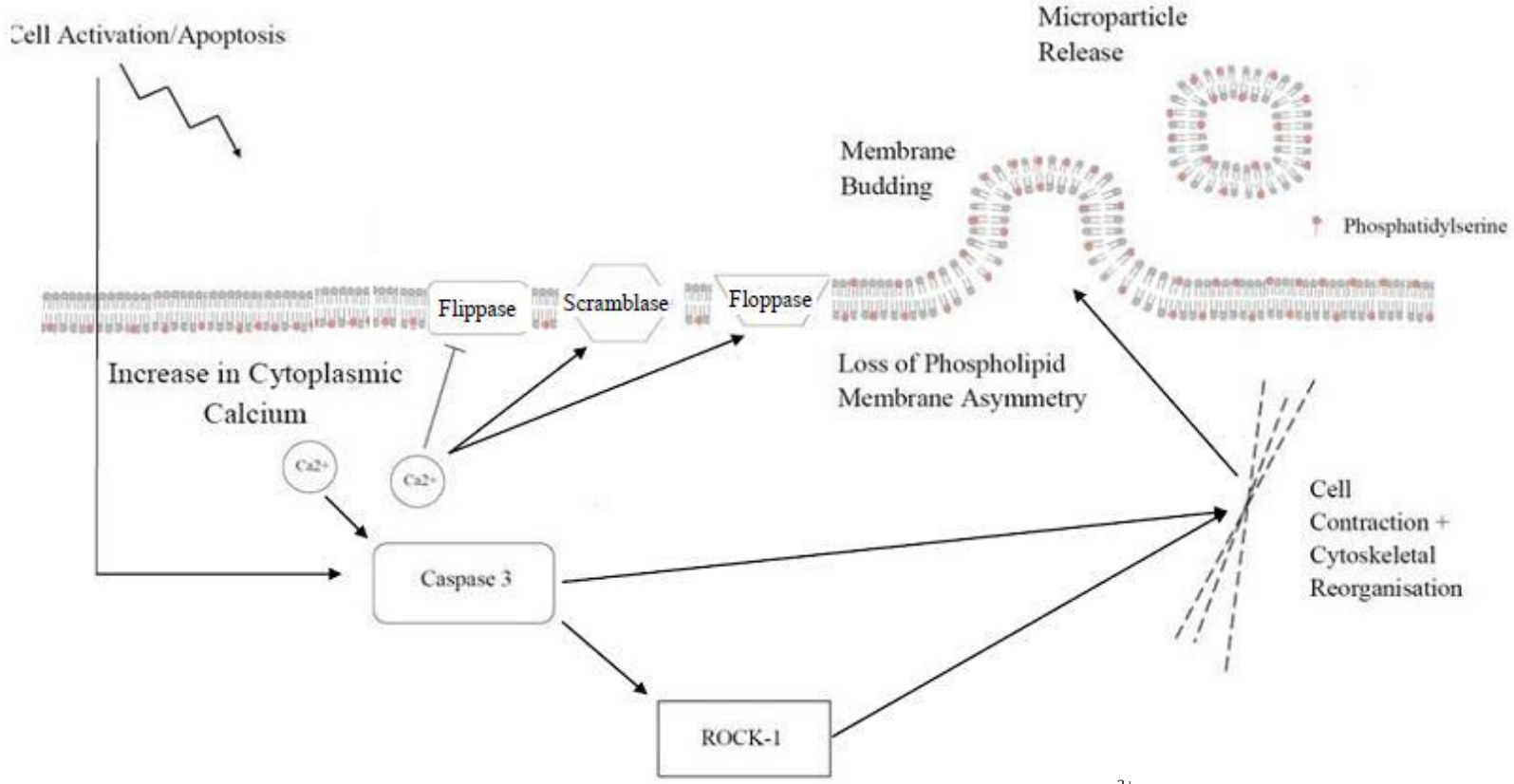


Figure illustrates the formation of microparticle release due to cell activation or cell apoptosis. Cytoplasmic Ca^{2+} increase due to the stimulus of activation or apoptosis causes the activation of enzymes scramblase and flippase whilst inhibiting floppase. This causes membrane phospholipid asymmetry to be lost and the negatively charged phospholipid phosphatidylserine to be exposed on outer membrane leaflet (one stimulus for membrane blebbing). Cytoplasmic Ca^{2+} increase can also cause the activation of caspase 3, which can directly or indirectly (via Rho-associated, coiled-coil-containing protein kinase [ROCK-1]) cause cell contraction and cytoskeletal reorganisation which results in microparticle formation.

2.5.4 Detection

Flow cytometry is the most common method used to enumerate MPs due to the rapidity of analysis. Flow cytometric analysis of MPs involves gating of 'events' which are <1μm in size, with further analysis for relevant surface antigen expression using fluorescent antibodies against EC markers. The antigens by which we can detect EMPs are: CD31, CD51, CD54, CD105, CD146, CD62E and CD144 (Shet *et al*., 2008; Chironi *et al*., 2009; Sossdorf *et al*., 2011). CD31 is also used to detect PMP, therefore when using CD31 to detect EMPs one must employ a platelet marker also (e.g CD42b) and EMPs are those expressing CD31 and not CD42b ($CD31^{+}CD42b^{-}$). Some investigators define MP as expressing the negatively charged phospholipid PS, which can be detected by the binding of the protein annexin V.

Interestingly EMPs formed by EC apoptosis have a higher expression of CD31, CD105 (Jimenez *et al*., 2003a) and CD144 (Simak *et al*., 2006), whereas those formed from EC activation have a higher expression of CD62E (Jimenez *et al*., 2003a), CD54 and CD106 (Combes *et al*., 1999; Jimenez *et al*., 2003a; Jimenez *et al*., 2005). Using this information, we may gain insights into the EC events that triggered MP release.

The sample is analysed cytometrically where a laser detects immunofluoresence emitted by fluorescent tags on antibodies attached to antigens on the cells/MPs. This produces quantifiable phenomenon called side scatter (SS) and forward scatter (FS). SS indicates granularity of the cells or particles, whereas FS indicates particle or cell size. There is a limitation to using flow cytometry in that detection of small MPs (<300nm) is limited by background noise (Ardoin *et al*., 2007).

No one standardised protocol exists to prepare blood samples for the flow cytometric detection of EMPs. This is because different laboratories employ different blood processing and preparation techniques. Some of the protocols currently being used are shown in table 2.2. Distinct common themes arise; (1) the use of the tourniquet should be minimised when taking the blood samples to minimise the risk of endothelial activation (Biro *et al*., 2004), and (2) the first 3mL should be discarded due to localised EMP release from the site of venepuncture (Shet *et al*., 2004). The majority of researchers also suggest that blood should be processed within 4 hours of blood sampling, as increasing the time from blood sampling to centrifugation leads to increased MP numbers (Ayers *et al*., 2011). In terms of blood processing, centrifugation of the sample at too high a speed may cause EMPs at the larger end of the scale to be spun out of the plasma thus rendering them unable to be analysed. This has been confirmed in experiments where $CD31^{+}CD42b^{-}$ EMPs were reduced after high speed centrifugation (van Ierssel *et al*., 2010). However, centrifugation at too low a speed may not eradicate platelets allowing for platelet activation and PMP formation during freezing and thawing of the sample (Dey-Harza *et al*., 2010). A wash protocol has been suggested to improve the signal: noise ratio, thus increasing specificity (Simak & Gelderman, 2006). However, this is labour intensive and may not be practical when dealing with incubations times and large sample volumes, which can be a potential source of error in analysis. Table 2.2 shows the most popular preparation techniques for MP analysis in human plasma. The table shows how there are double-centrifugation steps involved in isolating platelet-free plasma (PFP). The first spin attempts to remove most of the large cells and platelets with minimal activation of these cells (which could generate MPs). The remainder of platelets need to be removed prior to freezing with a second, more vigorous spin.

Table 2.2. Overview of Popular Plasma Processing Methods when Measuring Microparticles.

PPP/PFP Method	Isolation of MP Pellet	Reference
	-	Dignat-George *et al*., 2004
	-	Freyssinet, 2003
1500 x g, 15 min	-	Werner *et al*., 2005
13 000 x g, 2 min	-	Faure *et al*., 2006
	-	Sabatier *et al*., 2002
	-	Chironi *et al*., 2006
200 x g, 10 min 1500 x g, 7 min	-	Jimenez *et al*., 2004
13 000 x g, 10 min	-	Shet *et al*., 2004
1550 x g, 20 min	18 000 x g, 30 min	Biro *et al*., 2004
	18 890 x g, 30 min	van Beers *et al*., 2009
160 x g, 10 min	-	Chirinos *et al*., 2005a
1000 x g, 8 min	-	Chirinos *et al*., 2005b
	-	Arteaga *et al*., 2006
1500 x g, 10 min	-	Preston *et al*., 2003
160 x g, 10 min 1000 x g, 10 min	-	Esposito *et al*., 2006
1550 x g, 20 min 10 000 x g, 10 min	-	van Ierssel *et al*., 2010

PPP-Platelet Poor Plasma, PFP- Platelet Free Plasma

The majority of laboratories use frozen plasma for MP analysis. The freeze-thaw cycle is of speculative interest. Trummer *et al* (2008) observed that levels of PMPs were reduced when samples were thawed on ice, when compared to room temperature or 37°C. However, for those interested in EMPs, thawing procedure had no significant effect on EMP levels. The authors of the study recommended that when thawing frozen plasma samples, to thaw at room temperature or at 37°C. In summary, freezing samples allows batch analysis, thus minimising inter-assay variability, which could be considerable since we are detecting events at the lower limit of detection of flow cytometers.

Some investigators suggest that we must define MPs as annexin V^+ as the binding of this protein indicates presence of negatively charged phospholipids such as PS. However, it has been shown that only a fraction of all MPs bind annexin V (Horstman *et al*., 2004a; Horstman *et al*., 2004b). Horstman *et al* (2004b) showed that 35 times more CD62E (endothelial marker) binds to EMPs than annexin V.

2.5.5 Endothelial Microparticles and Disease States

EMPs are elevated in a variety of disease states including cardiovascular disease, preeclampsia, and cancer. Details of studies investigating EMPs in disease states are contained in table 2.3. It appears that EMPs may be a useful biomarker in these disease states involving endothelial damage and activation (Jimenez *et al*., 2005). These processes have been difficult to monitor due to the inaccessibility of the endothelium (Ahn, 2005).

Table 2.3. Endothelial Microparticles in Disease States.

Category	Disease State	Microparticle Definition	↑ / ↓	Reference
Cardiovascular Disease	ACS	$CD31^+CD41^-$ EMP	↑	Bernal-Mizrachi *et al*., 2004
	(MI, UA, SA, CHF)	$CD62E^+$ EMP	↑	Lal *et al*., 2009
		$CD31^+$Annexin V^+ EMP	↑	Werner *et al*., 2006
	CAD/ATH	$CD31^+$ AnnnexinV^+ EMP	↑	Sinning *et al*., 2010
	ED	$CD31^+$ EMP	↑	Arteaga *et al*., 2006
		$CD51^+$ EMP	↑	Arteaga *et al*., 2006
	MetS	Annexin V^+CD144^+ EMP	↑	Helal *et al*., 2010
Diabetes Mellitus	T1DM	$CD51^+$ EMP	↑	Sabatier *et al*., 2002
	T2DM	$CD144^+$ EMP	↑	Koga *et al*., 2005
Hypertension and Preeclampsia	HT	$CD31^+CD42^-$ EMP	↑	Preston *et al*., 2003
		$CD31^+CD42^-$ EMP	↑	Gonzalez-Quintero *et al*., 2004
		$CD62E^+$ EMP	↑	Gonzalez-Quintero *et al*., 2004
	PE	$CD31^+CD42^-$ EMP	↑	Gonzalez-Quintero *et al*., 2003

Table 2.3. Endothelial Microparticles in Disease States (continued).

Category	Disease State	Microparticle Definition	↑ / ↓	Reference
Inflammatory Disorders	Vasculitis	$CD105^+$ EMP	↑	Brogan *et al*., 2004
Autoimmune Disorders	LA	$CD31^+CD51^+$ EMP	↑	Combes *et al*., 1999
	APS	$CD31^+$ EMP	↑	Morel *et al*., 2005
Haematological Disorders	TTP	$CD31^+$ EMP	↑	Jimenez *et al*., 2001
		$CD51^+$ EMP	↑	Jimenez *et al*., 2001
		$CD62E^+$, $CD31^+CD42b^-$ EMP	↑	Jimenez *et al*., 2003b

ACS- Acute Coronary Syndrome, APS-Anti-Phospholipid Syndrome, ATH- Atherosclerosis, CAD- Coronary Artery Disease, CHF- Congestive Heart Failure, ED- Endothelial Dysfunction, HT- Hypertension, LA- Lupus Anticoagulant, MetS- Metabolic Syndrome, MI- Myocardial Infarction, MS- Multiple Sclerosis, PE- Preeclampsia, SA- Stable Angina, T1DM- Type 1 Diabetes Mellitus, T2DM- Type 2 Diabetes Mellitus, TTP- Thrombotic Thrombocytopenic Purpura, UA- Unstable Angina, EMP-Endothelial Microparticles, PMP- Platelet Microparticles, GMP- Granulocyte Microparticles. Adapted from Jimenez et al., 2005

2.5.6 Endothelial Microparticles and Angiogenesis

EMPs that harbour MMP-2 and MMP-9 in their membranes may assist in angiogenesis (Taraboletti *et al*., 2002). MMPs are crucial to angiogenesis whereby degradation of the endothelial basement membrane is critical in order for ECs to proliferate and migrate to generate a lumen. Leroyer *et al* (2009) showed that EMPs were higher in ischemic hind limb muscles in mice than in control mice, and these EMPs stimulated EC proliferation *in vitro*. The mRNA encapsulated within EMPs has been shown to stimulate Akt pathway-dependent angiogenesis in ECs (Deregibus *et al*., 2007). However, EMPs have also been observed to impair cell proliferation in human umbilical vein ECs (HUVEC) on a Matrigel substrate (Mezentsev *et al*., 2005). These authors observed a reduction in EC proliferation rate in the presence of EMPs and also an increase in HUVEC apoptosis. EMPs have also been shown to interfere with the functioning of circulating angiogenic cells (CAC) by inducing apoptosis (Distler *et al*., 2011). It may be that the level of circulating EMPs in circulation may determine whether there is a pro-angiogenic or anti-angiogenic effect, with pathophysiologically high concentrations inhibiting angiogenesis (Mezentsev *et al*., 2005), and low concentrations promoting angiogenesis (Taraboletti *et al*., 2002).

2.5.7 Putative Roles of Microparticles

EMPs may not simply be passive end-products in processes, but may also regulate cellular pathways, thereby having a role in biological processes. One possible function of MPs is communication and the transfer of packaged biological material between cells. MPs are believed to be able to transfer antigens to epithelial cells (Mesri & Altieri, 1998), chemokines to ECs leading to upregulation of adhesion molecule

expression (Nomura *et al*., 2011), mRNA to other cells (Deregibus *et al*., 2007; Ullal *et al*., 2010), may cause blood vessel damage (Boulanger *et al*., 2001; VanWijk *et al*., 2002), communicate to other MPs through protein transfer (Horstman *et al*., 2004a), and may also produce reactive oxygen species (ROS) (Mezentsev *et al*., 2005; Leroyer *et al*., 2009). The most interesting role may be the transfer of genetic material (e.g. DNA, mRNA or miRNA). The transfer of genetic material could have a profound effect in the regulation of gene expression altering cellular functional output.

Another possible function of MPs is cell defence and preservation. It may be that MPs are released in order to prevent cell death, thus suggesting a novel early defence mechanism. Cells may release toxic materials and/or negatively charged phospholipids (which are pro-coagulant and pro-thrombotic) in the form of MPs in order to prevent further damage to the parent cell, and perhaps to other cells (Freyssinet & Toti, 2010).

2.5.8 Exercise and Endothelial Microparticles

A number of studies have investigated the effects of exercise on MPs. Some (Sossdorf *et al*., 2010; Chaar *et al*., 2011; Sossdorf *et al*., 2011) but not all studies (Chen *et al*., 2010) observe an increase in PMPs, suggesting platelet activation. One study has found a significant increase in EMPs as a result of aerobic exercise in trained individuals (Sossdorf *et al*., 2011). However the majority of studies have repeatedly found no changes in circulating EMPs in trained and untrained individuals due to bouts of aerobic exercise (Möbius-Winkler *et al*., 2009; Chen *et al*., 2010; Sossdorf *et al*., 2010; Chaar *et al*., 2011) suggesting platelet-specific activation during exercise due to increased levels of shear stress. However, one study found no increase in PMP levels after an intense bout of exercise (Chen *et al*., 2010).

Möbius-Winkler *et al* (2009) investigated the effect of prolonged cycling exercise of moderate intensity (70% of their anaerobic threshold) on EMPs in healthy individuals. EMPs were defined as $CD62E^{+}CD42b^{-}$, and were found to be unchanged throughout the 4 hour exercise period, as well as throughout the 24 hour recovery period.

Sossdorf *et al* (2011) studied the effects of short duration (90 minutes) cycling exercise of moderate intensity (80% of their anaerobic threshold) on circulating MPs in trained and untrained individuals. EMPs were defined as $CD62E^{+}$, PMPs defined as $CD42a^{+}$, and monocyte-derived MPs defined as $CD14^{+}$ events. MPs were also assessed for the presence of PS on the MP membrane through binding of annexin V. Procoagulant activity of circulating MPs was also measured. There were no differences in MP numbers between the trained and untrained individuals at rest. There was an increase in annexin V^{+} MPs post-exercise, with the highest level at 45 minutes post-exercise in trained individuals, and 2 hours in untrained individuals. PMPs, monocyte-derived MPs and EMPs were all increased in trained individuals post-exercise, with only PMP increasing in untrained individuals. Procoagulant activity of MPs was also increased immediately after exercise. In a similar study, Sossdorf *et al* (2010) investigated the same bout of exercise in 16 healthy male volunteers and found increases in $CD42b^{+}$ PMPs with no other increases in other MP subsets (EMP or monocyte-derived MP). Again, procoagulant activity of MPs was increased post-exercise. The authors postulated that there was an increase in PMPs as 'juvenile' platelets were released into the circulation from the bone marrow (BM) and spleen, and that these platelets were more susceptible to activation and MP release.

Chen *et al* (2010) examined the influence of a graded exercise test on PMPs as well as shear stress-mediated thrombin generation by PMPs from plasma both pre- and post-

exercise in sedentary healthy men. PMPs, identified by CD61 positivity were enumerated by flow cytometry. There was no increase in circulating PMPs pre- and post-exercise. Plasma from these subjects pre- and post-exercise was also exposed to low and high levels of shear stress. High levels of shear stress applied to post-exercise plasma produced an increase in PMPs in these subjects.

Chaar *et al* (2011) also investigated the effects of exercise on MP release in healthy male subjects. The exercise mode consisted of intermittent submaximal and maximal exercise bouts (mimicking a typical training session in intermittent sports). ErMPs ($CD235a^+$), PMPs ($CD41^+$), neutrophil-derived MPs ($CD15^+$), monocyte-derived MPs ($CD14^+$) and EMPs ($CD106^+$) were enumerated by flow cytometry. Both PMPs (immediately post-exercise) and neutrophil-derived MPs (immediately and 2 hours post-exercise) were increased as a result of the intermittent exercise bout. However, no changes in the other MP subtypes was observed. These results were accompanied by increases in IL-6 levels, suggesting a significant inflammatory response. The lack of EMP release as a result of exercise suggests no significant endothelial activation or apoptosis occurred.

In summary EMPs have yet to be consistently found to be released due to an exercise bout, whereas PMPs have consistently been shown to be increased, suggesting platelet-specific activation as a result of exercise-induced increase in shear stress.

2.6 Endothelial Progenitor Cells

The term ‘progenitor’ refers to a population of cells that are in a dormant state (Potten & Loeffler, 1990) in storage areas of the body, but have the ability to enter the cell cycle on demand and are able to differentiate into specialised phenotypes including EC

lineage (Devanesan *et al*., 2009). Unlike stem cells, progenitor cells have limited self renewal ability whereas stem cells have unlimited ability to replicate.

Endothelial progenitor cells (EPC) were first identified in 1997 (Asahara *et al*., 1997). They were first described as $CD34^{+}VEGFR2^{+}$ cells which differentiate into ECs *in vitro* which could contribute to angiogenesis and neovascularisation in ischemic tissues in animals (Asahara *et al*., 1997). The term EPC is used to describe different cell types which contribute to endothelial repair and growth in different ways, with some 'true' progenitors differentiating into ECs and taking residence in the endothelium, and others secrete angiogenic factors which act on ECs to proliferate.

Since 1997 there has been considerable developments in EPC knowledge. The original definition of EPC used by Asahara is now believed to encompass cell types of different origin, lineage and function, but contributing to endothelial growth and repair by different mechanisms (Pearson, 2009). A small number may be 'true' progenitors, with these cells differentiating into ECs and taking residence into the endothelium, and the remainder secreting angiogenic factors which act on ECs to proliferate.

EPCs are primarily produced and reside in the BM (Lyden *et al*., 2001; Devanesan *et al*., 2009) but also reside in other storage areas of the body such as the liver, epicardium, intestine (Devanesan *et al*., 2009) and the spleen (Asahara *et al*., 1999b; Laufs *et al*., 2004), with a small number in the circulation acting to repair and maintain vascular integrity (Masuda & Asahara, 2003; Adams *et al*., 2004; Laufs *et al*., 2004; Garmy-Susini & Varner, 2005; Sata, 2006; Werner & Nickenig, 2006; Balestrieri *et al*., 2008; Kirton & Xu, 2010; Pesce *et al*., 2011). They are however rare events in human

peripheral blood, accounting for between 0.0001 and 0.01% of all mononuclear cells (Case *et al*., 2007).

2.6.1 Mobilisation, Homing and Differentiation of Endothelial Progenitor Cells

In order for EPCs to exert its effects on the endothelium, they must first be mobilised from their storage areas within the body. The mobilisation of EPCs occurs largely due to increased levels of cytokines and growth factors, such as VEGF (Asahara *et al*., 1999a; Kalka *et al*., 2002a; Kalka *et al*., 2002b; Heiss *et al*., 2010), stromal cell-derived factor-1 (SDF-1) (Hattori *et al*., 2001; Yamaguchi *et al*., 2003; Heiss *et al*., 2010), basic fibroblast growth factor (bFGF) (Gnecchi *et al*., 2008) and granulocyte colony stimulating factor (G-CSF) (Kalka *et al*., 2002b) as described in figure 2.8. These mobilising factors are released in ischemia (Pillarisetti & Gupta, 2001), hypoxia (Shweiki *et al*., 1992; Olfert *et al*., 2001; Ceradini *et al*., 2004) and in the presence of high circulating NO levels (Aicher *et al*., 2003; Laufs *et al*., 2004). Indeed EPCs (expressing VEGFR2) were unable to migrate towards VEGF under the inhibition of NOS (Heiss *et al*., 2010).

In the BM, progenitor cells such as EPCs are bound to stromal cells (Urbich & Dimmeler, 2004). In order for progenitor release from the BM, this association must be disrupted, thus allowing for progenitors to be released into the circulation (Urbich & Dimmeler, 2004). This process involves the above named growth factors and cytokines, as well as proteinases such as elastase and MMPs. MMP-9 has a very important role in cleaving the bond between EPCs and stromal cells (Takahashi *et al*., 1999; Heissig *et al*., 2002) as well as cleaving EPC membrane-bound Kit ligand (KitL or stem cell factor

[SCF]). KitL also tethers EPCs to BM stromal cells (Kodama *et al*., 1994), and its conversion to soluble KitL allows EPCs to migrate into the circulation (Heissig *et al*., 2002; Kirton & Xu, 2010). G-CSF is thought to induce mobilisation indirectly through the release of elastase and cathepsin G from neutrophils (Aicher *et al*., 2005). These proteinases may cleave EPC bonds to either stromal cells or by cleaving EPC membrane-bound KitL. The chemokine SDF-1 may induce mobilisation through chemoattraction of CXC-chemokine receptor 4 (CXCR4) expressing EPCs into the circulation. It is the binding of ligand with its receptor which ensures that EPCs reside on the BM vascular endothelium (Peled *et al*., 1999; Ceradini *et al*., 2004). $CXCR4^+$ EPCs can be mobilised by firstly the dissociation of ligand and its receptor in the BM (Devine *et al*., 2004; Jujo *et al*., 2010) followed by an increased level of circulating SDF-1 which will cause $CXCR4^+$ EPCs to circulate in the blood (Hattori *et al*., 2001). G-CSF may play a role in the dissociation of BM endothelium expressing SDF-1 with EPC-bound CXCR4 via proteinase release (Gomes *et al*., 2010). EPCs have been found to migrate towards a gradient of SDF-1 *in vitro* in a dose-dependent manner (Heiss *et al*., 2010). Other mobilising factors include oestrogen (Strehlow *et al*., 2003), erythropoietin (Heeschen *et al*., 2003) and statins (Pistrosch *et al*., 2005; Werner *et al*., 2007).

Once in the circulation EPCs need to 'home' to sites of endothelial damage and growth. Very little is known regarding how EPCs 'home' to sites where they are required, however, some believe that integrins play a key role. SDF-1, released from ECs, can be a chemoattractant to these sites of ischemic tissue or vascular regeneration. SDF-1 plasma levels can be increased as a result of increased SDF-1 gene expression in ECs (Sung *et al*., 2009). In ischemic tissues, levels of hypoxia are related to levels of SDF-1

expression in ECs indicating that SDF-1 is produced in ischemic tissues and plays a role in homing $CXCR4^+$ EPCs (Ceradini *et al*., 2004; Ceradini & Gurtner, 2005).

When the EPCs are at the site of endothelial damage or growth, they may differentiate into the EC lineage (Asahara *et al*., 1997; Werner & Nickenig, 2006). In order for EPCs to differentiate, they must first adhere to the EC wall (Chavakis *et al*., 2005). EPCs may adhere to the endothelial wall via vascular adhesion molecules, e.g. ICAM-1 and VCAM-1, both of which are found on EC membranes (Friedman *et al*., 1986; Korenaga *et al*., 1997) and β2 integrins (Chavakis *et al*., 2005). SDF-1 can also be upregulated on the EC wall to attract $CXCR4^+$ EPCs to the endothelium (Ceradini *et al*., 2004), and has been shown to promote EPC incorporation into ischemic vasculature in a dose-dependent manner (Yamaguchi *et al*., 2003). The process of differentiation into ECs can be measured by the increased expression of endothelial protein markers on EPC membranes. Differentiation has been shown to be mediated by shear stress *in vitro* (Ye *et al*., 2008; Obi *et al*., 2009) and by exposure to EC-derived NO (Chu *et al*., 2008).

Figure 2.8. Mobilisation of EPCs from the Bone Marrow.

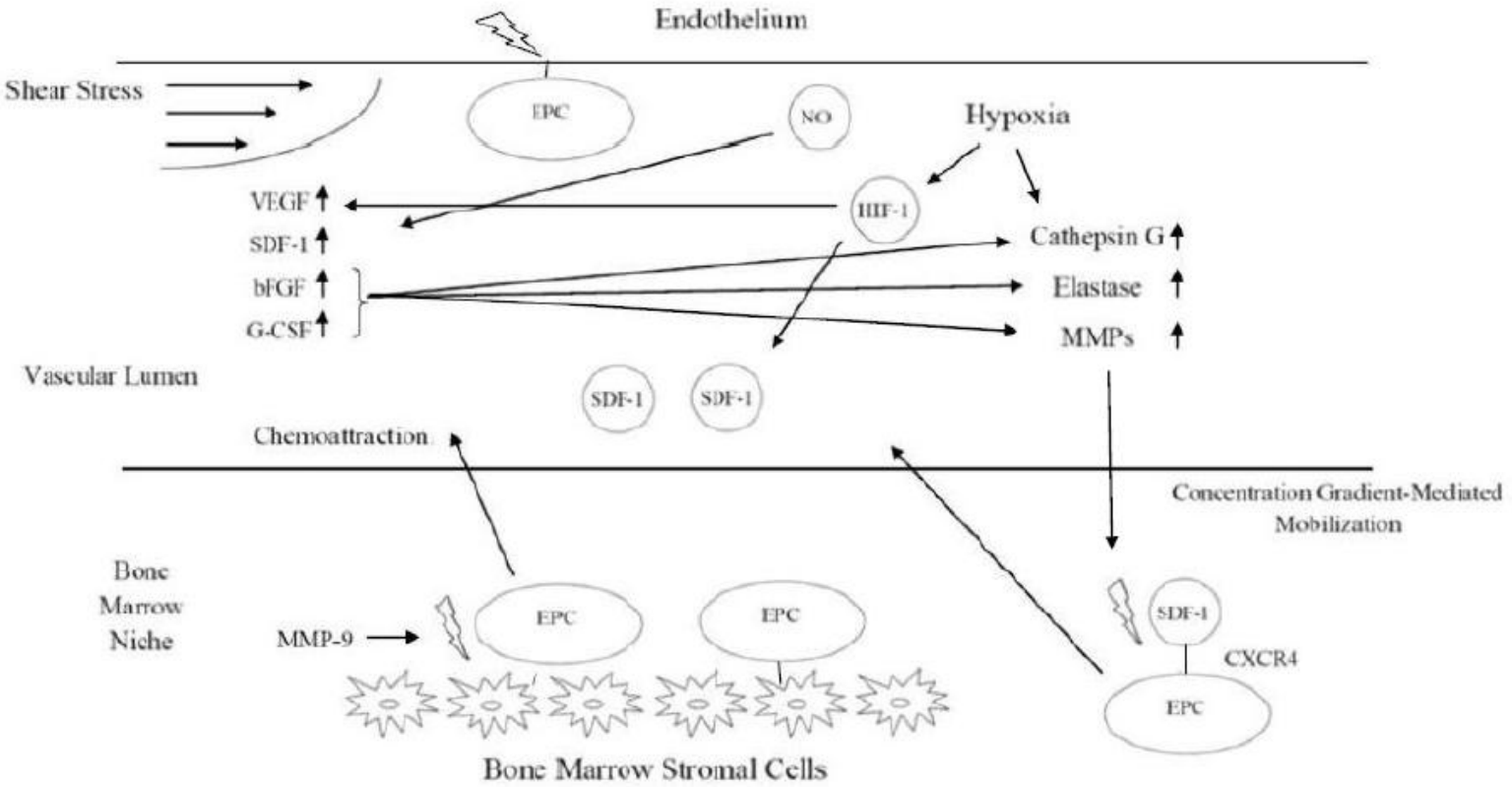


Figure illustrates some of the pathways by which EPCs are mobilised from the bone marrow (BM) or from the endothelial cell (EC) wall. VEGF= Vascular endothelial growth factor, SDF-1= Stromal derived factor-1, bFGF= basic fibroblast growth factor, G-CSF= Granulocyte-colony stimulating factor, NO= Nitric oxide, HIF-1= Hypoxia-inducible factor-1, MMP-9= Matrix metalloproteinase-9, CXCR4 = CXC Chemokine Receptor 4. Adapted from Hristov et al., 2003a

2.6.2 Endothelial Progenitor Cells in Angiogenesis and Neovascularisation

The role of EPCs in the growth of new blood vessels has been at the forefront of EPC research in the last 15 years. There is debate as to how EPCs may help in the growth or repair of blood vessels. There are 2 possible ways in which EPCs may play a role in the growth of new blood vessels:

1. Angiogenesis

2. Neovascularisation

For the remainder of this thesis, angiogenesis will describe the process of new blood vessel growth due to the sprouting of ECs or the splitting of an existing blood vessel into two new vessels. EPCs may play a part in this process through secretion of pro-angiogenic factors which promote the proliferation of ECs (Urbich & Dimmeler, 2004). Neovascularisation refers to the process by which precursor cells from the BM (e.g. EPCs) incorporate into the endothelium and differentiate into mature ECs (Masuda & Asahara, 2003).

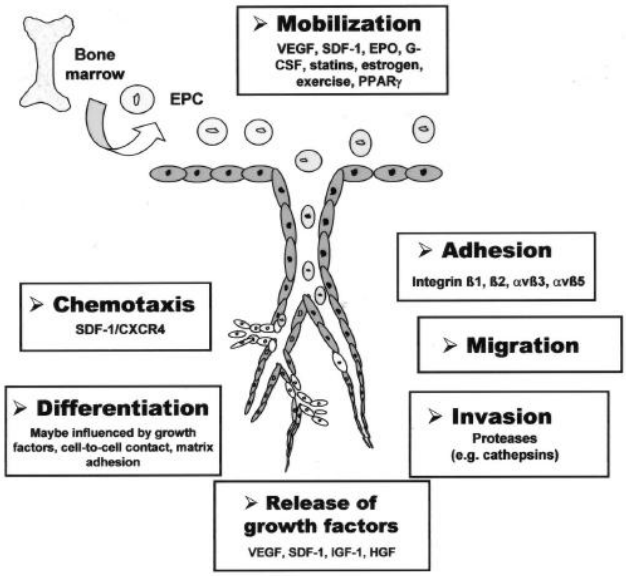
There is evidence for both. EPCs can support angiogenesis through the release of angiogenic factors such as VEGF, insulin-like growth factor-1 (IGF-1), hepatic growth factor (HGF) (Urbich & Dimmeler, 2004) and platelet-derived growth factor (PDGF) (Urbich *et al*., 2005; Gnecchi *et al*., 2008; Di Santo *et al*., 2009; Wyler von Ballmoos *et al*., 2010). These growth factors mediate the proliferation of ECs that reside in the blood vessel in question (Urbich *et al*., 2005). EPCs too can directly incorporate into the EC

wall and differentiate into ECs. They have been found to directly incorporate into sites of blood vessel growth in animal models (Asahara *et al*., 1997; Patterson, 2003) and can form tube structures *in vitro* (Hur *et al*., 2004; Rae *et al*., 2011). However numerous studies have found that EPCs incorporating into ischemic tissues to be quite low (Rajantie *et al*., 2004; Urbich & Dimmeler, 2004; Peters *et al*., 2005). Murayama *et al* (2002) on the other hand reported that 25% of cells making up newly formed blood vessels were EPC-derived *in vivo* when measured by immunofluorescence. However this number fell to 5.7% when analysing with X-gal staining of a Matrigel plug highlighting the point that measuring EPC differentiation into EC lineage by different methods gives different results.

It may be that the different subtype of EPC may determine whether they exert a proangiogenic effect through secretion of growth factors or they incorporate directly into the tissue for neovascularisation. Early outgrowth cells (EOC), a subset of EPC that appears early in culture, have been found to act in a paracrine manner on ECs (Sieveking *et al*., 2008) by secreting a high amount of proangiogenic factors (Hur *et al*., 2004) and they fail to proliferate into colony forming units (CFU) in endothelial growth medium (Case *et al*., 2007). Late outgrowth cells (LOC), another subset of EPC which appears late in culture, have the ability to proliferate and differentiate into ECs (Hur *et al*., 2004; Yoon *et al*., 2005; Mukai *et al*., 2008) but have limited ability to secrete proangiogenic factors (Hur *et al*., 2003; Rehman *et al*., 2003; Urbich *et al*., 2003). In summary, it seems that EPCs play a direct role in blood vessel growth and repair by incorporating into the endothelium (LOC), as well as supporting the process through the release of pro-angiogenic growth factors which stimulate the proliferation and migration of ECs (EOC). These EPC subsets provides an opportunity to develop cell therapies for

those who suffer tissue ischemia as well as a possible mechanism for exercise-induced blood vessel growth.

Figure 2.9. EPCs Involvement in Blood Vessel Growth.



Urbich & Dimmeler, 2004

2.6.3 Detection

EPCs are enumerated by flow cytometry. EPC function is typically evaluated by culturing mononuclear cells which gives rise to both early and late outgrowth colonies depending on the culture technique used. For this EPCs can be defined as peripheral blood MNC (PBMNC) which can be extracted from a whole blood sample. They can be identified by their ability to adhere to fibronetin to take up acetylated low-density lipoprotein (acLDL) and bind Ulex lectin (Asahara *et al*., 1997). EPC enumeration by flow cytometry is complicated by their low number in the peripheral circulation (Dulic-Sills *et al*., 2006).

With flow cytometry one needs to define cells of interest by their protein markers. The most common way to identify EPCs is by immunolabelling with one progenitor/stem cell marker and one endothelial marker. EPCs express many endothelial protein markers such as VEGFR2, von Willebrand factor (vWf) and vascular endothelial cadherin (VE-cadherin/CD144) (Hristov *et al*., 2003b; Urbich & Dimmeler, 2004; Rae *et al*., 2011). They also express markers of stem cell lineage such as CD34, CD133 and CD177 (Hristov *et al*., 2003b; Urbich & Dimmeler, 2004; Rae *et al*., 2011). Reportedly EPCs make up only ~4.5% of all $CD34^+$ stem cells and are small in size, as indicated by low SS and FS in flow cytometry (Burger *et al*., 2002). The most consistently used definitions of EPCs as identified by flow cytometry are $CD34^+VEGFR2^+$ and $CD34^+CD133^+VEGFR2^+$. $CD34^+VEGFR2^+$ cells have been consistently observed to be good indicators of cardiovascular risk (Schmidt-Lucke *et al*., 2005; Fadini *et al*., 2008a).

It has been suggested in order to distinguish between LOCs and EOCs we can use the pan-hematopoietic marker CD45, whereby cells which are positive for CD45 are EOC (Urbich *et al*., 2003) and those cell which are negative or dimly expressing CD45 are LOCs (Ingram *et al*., 2005). Some argue that CD45 is expressed solely on leukocytes and not in EPCs in peripheral blood, as $CD34^+CD45^-$ cells have good endothelial CFU ability, whereas $CD34^+CD45^+$ cells do not (Case *et al*., 2007). However Urbich *et al* (2003) previously demonstrated that CACs which formed CFUs expressed CD45. Therefore during flow cytometric analysis it would be advantageous to gate $CD34^+$ cells which are low in SS and FS, then both $CD45^-$ and $CD45^+$ populations and subsequently gate $VEGFR2^+$ cells to identify any possible definition of EPCs.

Pujol *et al* (2000) also suggests the use of CD14 as a marker of EPCs. In disagreement $CD14^+$ and $CD14^-$ PBMNCs have shown equal expression of endothelial markers and have similar ability to form CFUs in endothelial medium, thus negating the need for analysis of EPCs by CD14 (Urbich *et al*., 2003).

Some recommendations for EPC detection (using the ISHAGE guidelines as a template) are as followed:

- When taking blood ethylene diamine tetraacetic acid (EDTA) anti-coagulant tubes are essential.
- Incubation of sample with monoclonal antibodies for 20-30 minutes followed by lysis.
- Lysis for at least 10-15 minutes.
- EPCs $CD34^+$ $VEGFR2^+$ whilst also measuring $CD45^+$ and $CD45^-$ subpopulations.
- Minimum of 100 $CD34^+$ events or 75000 $CD45^+$ events to be detected during flow cytometric analysis.
- Absolute leukocyte count to be obtained from haematology analyser to determine EPC count per volume of blood.

2.6.4 Endothelial Progenitor Cells and Disease States

Unlike most cardiovascular biomarkers, EPCs are often lower in peripheral circulation of those with cardiovascular disease (Fadini *et al*., 2006a), those with cardiovascular risk factors including smoking (Vasa *et al*., 2001), diabetes (Sibal *et al*., 2009) and inflammatory disorders (Zhu *et al*., 2006) compared to age-matched healthy controls. Details are given in table 2.4. EPCs may be a possible biomarker of cardiovascular risk as these lower levels of EPCs are independent predictors of future cardiovascular events

(Schmidt-Lucke *et al*., 2005). The reduction in EPC numbers in circulation in these various disease states may be due to a reduced ability to mobilise the EPCs from the storage areas of the body due to reduced levels of mobilising factors (Fadini *et al*., 2006b; Heiss *et al*., 2010; Jialal *et al*., 2010). In disease states, especially with those with a reduced thickness in the endothelial cell's glycocalyx layer, may not be able to produce NO (a known mediator of EPC release) in response to shear in amounts required to mobilise EPCs. There may also be increased senescence of the EPCs leading to reduced viable EPC levels in circulation. Increased demand for vascular repair may also deplete the EPC pool.

Table 2.4. Endothelial Progenitor Cell Number and Function in Disease States.

Category	Disease	EPC Definition	↑ / ↓	Reference
Cardiovascular Disease	ATH	$CD34^+VEGFR2^+$	↓	Fadini *et al*., 2006a
	ED	$CD31^+VEGFR2^+$ EC-CFU	↓	Hill *et al*., 2003
Cardiovascular Risk Factors	HC	$CD31^+VEGFR2^+$ EC-CFU	↓	Hill *et al*., 2003
	HT	$CD31^+VEGFR2^+$ EC-CFU	↓	Hill *et al*., 2003
	SM	$CD34^+VEGFR2^+$ $CD34^+VEGFR2^+CD133^+$	↓	Vasa *et al*., 2001
Diabetes Mellitus	DM	$CD31^+VEGFR2^+$ EC-CFU	↓	Hill *et al*., 2003
	T1DM	$CD34^+CD144^+$ $CD133^+CD144^+$ $CD133^+VEGFR2^+$	↓	Sibal *et al*., 2009
	T2DM	$CD34^-CD133^+VEGFR2^+$	↓	Jung *et al*., 2010
Inflammatory Disorders	HHc	$CD133^+VEGFR2^+$	↓	Zhu *et al*., 2006

ATH-Atherosclerosis, EC-CFU-Endothelial Cell Colony Forming Units, ED-Endothelial Dysfunction, DM-Diabetes Mellitus, HC-High Cholesterol, HHc-Hyperhomocystenemia, HT- Hypertension, SM-Smoking, T1DM-Type 1 Diabetes Mellitus, T2DM-Type 2 Diabetes Mellitus

2.6.5 Age, Gender and Endothelial Progenitor Cells

Age and gender also influence EPC number and function. EPC function as measured by CFU or migratory capacity is lower in middle aged and older men when compared to younger individuals (Hoetzer *et al*., 2007), as well as their ability to secrete pro-angiogenic factors (Kushner *et al*., 2010). EPC senescence is increased with age as measured by reduction in telomere length (Hoetzer *et al*., 2007; Kushner *et al*., 2009) and activity (Kushner *et al*., 2011).

Gender also appears to have an effect on both number and functional ability of EPCs. It seems that women have higher circulating levels and better function of EPCs than men (Fadini *et al*., 2008b) thought to be due to oestrogen stimulated mobilisation of these cells (Fadini *et al*., 2009). In fact, testosterone had no effect on EPC number and functionality. These results suggest a gender-specific androgen effect on EPC mobilisation.

2.6.6 Exercise and Endothelial Progenitor Cells

EPCs are important in physiological health therefore mechanisms ensuring their numbers and functionality remain high are essential. Exercise is a lifestyle intervention which has been postulated to improve cardiovascular health. Research is currently focusing on the effects of acute and chronic exercise, as well as physical inactivity on EPCs.

Both acute (Adams *et al*., 2004; Rehman *et al*., 2004; Laufs *et al*., 2005; Van Craenenbroeck *et al*., 2008; Goussetis *et al*., 2009; Möbius-Winkler *et al*., 2009; Thorell

et al., 2009; Bonsignore *et al*., 2010; Sandri *et al*., 2011; Van Craenenbroeck *et al*., 2011) and chronic (Laufs *et al*., 2004; Sandri *et al*., 2005; Steiner *et al*., 2005; Hoetzer *et al*., 2007; Schlager *et al*., 2011) exercise has been shown to increase EPC number and function (Jenkins *et al*., 2009). However there are few studies which report no change following acute (Shaffer *et al*., 2006; Adams *et al*., 2008; Van Craenenbroeck *et al*., 2010) or with chronic exercise (Thijssen *et al*., 2006; Manfredini *et al*., 2009). In one study exercise training has been shown to reduce circulating EPC number in old men (Taddei *et al*., 2000). Summary of acute exercise and exercise training studies and EPCs are detailed in table 2.5 and table 2.6 respectively.

Table 2.5. Influence of Acute Exercise Studies on Endothelial Progenitor Cell Number and Function

Form of Exercise	Subject Characteristics	♂/♀	EPC Definition	Timepoints Measured	Result	Reference
Maximal exercise stress test	CAD patients with and without MI + healthy subjects	Not Given	$CD34^{+}VEGFR2^{+}$	Pre-, 2h, 4h, 6h, 8h, 24h, 48h, 72h, 96h, 120h, and 144h post-exercise	Increase in CAD patients with MI	Adams *et al*., 2004
Symptom-limited treadmill or bicycle exercise test	Male and female healthy volunteers	16♂ 6♀	$CD133^{+}CD144^{+}$	Pre- and 10 min post-exercise	Increase	Rehman *et al*., 2004
30 minutes @ 82% $\dot{V}O_2$ max 30 minutes @ 68% $\dot{V}O_2$ max 10 min @ 68% $\dot{V}O_2$ max	25 healthy volunteers	25♂	EPC	Pre- and 10 min and 30 min post-exercise	Increase in EPCs during 30min running @ 82% and 68% $\dot{V}O_2$ max	Laufs *et al*., 2005

CAD = Coronary Arterial Disease, EPC = Endothelial Progenitor Cell, MI = Myocardial Ischemia, ♂ =male subjects, ♀=female subjects.

Table 2.5. Influence of Acute Exercise Studies on Endothelial Progenitor Cell Number and Function (continued).

Form of Exercise	Subject Characteristics	♂/♀	EPC Definition	Timepoints Measured	Result	Reference
Maximal treadmill exercise test	9 young, 13 older healthy subjects +15 PAD patients	Not Given	$CD133^+VEGFR2^+$	Pre- and 10 min post-exercise	No change	Shaffer *et al*., 2006
			$CD34^+VEGFR2^+$		No change	
			$CD133^+CD34^+VEGFR2^+$		No change	
			$CD133^+CD34^+VEGFR2^+CD31^-$		Decrease in PAD and older subjects	
			$CD34^+VEGFR2^+CD146^-CD31^-$		No change	
Symptom-limited cardiopulmonary exercise test on bicycle ergometer	Healthy volunteers No CV risk factors	6♂, 5♀ 9♂, 5♀	$CD34^+VEGFR2^+$	Pre- and 10 in post-exercise	Increase	Van Craenenbroeck *et al*., 2008
246km foot race	10 volunteer athletes	10♂ assumed	EPC CFU-EC	Pre-, immediately post- and 48h post-exercise	Increase	Goussetis *et al*., 2009
4hr cycling @ 70% IAT	18 healthy young men	18♂	$CD34^+VEGFR2^+$	Pre- during, 30, 60, 120, 240, 1440 min post-exercise	Increase during and immediately post- exercise	Möbius-Winkler *et al*., 2009

CV = Cardiovascular, EC-CFU = Endothelial Cell Colony Forming Units, EPC = Endothelial Progenitor Cell, IAT = Individual Anaerobic Threshold, PAD = Peripheral Arterial Disease, ♂=male subjects, ♀ =female subjects.

Table 2.5. Influence of Acute Exercise Studies on Endothelial Progenitor Cell Number and Function (continued).

Form of Exercise	Subject Characteristics	♂/♀	EPC Definition	Timepoints Measured	Result	Reference
1hr bicycle spinning exercise	11 healthy volunteers	2♂, 9♀	LOC Colonies	Pre-, 1hr, 6hr, 24hr, 48hr post-exercise	Increase 1hr post-exercise	Thorell *et al*., 2009
Marathon race 1.5km run	10 healthy amateur runners	10♂	$CD34^+VEGFR2^+$ $CD133^+CD144^+$	Pre- immediately post- and 20hr post- marathon Pre- and immediately post- 1.5km race	Increase immediately post- marathon and immediately post 1.5km race	Bonsignore *et al*., 2010
Cardiopulmonary exercise test	41 CHF patients 13 healthy subjects	33♂, 8♀ 9♂, 4♀	$CD34^+VEGFR2^+$	Pre- and post-exercise	No change	Van Craenenbroeck *et al*., 2010
Maximal treadmill exercise test	23 PAOD patients	22♂, 1♀	$CD34^+VEGFR2^+$	Pre- and up to 72hr post-exercise	Increase with max at 24hr post- and baseline by 72hr post-exercise	Sandri *et al*., 2011
Symptom-limited graded bicycle ergometer exercise test	7 CHF patients 4 young and 4 older healthy subjects	5♂, 2♀ 8♂	$CD34^+VEGFR2^+$	Pre-, 10 min, 30 min, 1, 2, 4, 8, 12, 24hr post-exercise	Increase in EPC in young and older healthy subjects.	Van Craenebroeck *et al*., 2011

CHF = Chronic Heart Failure, EPC = Endothelial Progenitor Cell, LOC = Late Outgrowth Cell, PAOD = Peripheral Arterial Occlusive Disease, ♂=male subjects, ♀=female subjects.

Table 2.6. Summary of Exercise Training Studies and Endothelial Progenitor Cells.

Form of Exercise	Subject Characteristics	♂/♀	EPC Definition	Result	Reference
Voluntary running in running wheel	Mice (eNOS-/- and wild-type)	n/a	Sca-1^{+}VEGFR2^{+} EPCs measured in mice	In mice, EPCs increased after 7 days voluntary running, sustained up to 28 days of training	Laufs *et al*., 2004
4 week bicycle ergometer training, moderate muscle strength training and regular walking	19 human CAD patients	9♂, 10♀	CD34^{+}VEGFR2^{+} EPCs in humans	In humans, EPCs increased after 28 days training	
4 weeks ischmic treadmill training	9 ischaemic PAOD patients	Not Given	CD34^{+}VEGFR2^{+} EPCs	Increase	Sandri *et al*., 2005
4 weeks non-ischemic treadmill training	9 non-ischaemic PAOD patients			No change	
4 weeks sub-ischemic bicycle ergometer training	15 stable CAD patients			No change	
12 week supervised running training	20 patients with CAD and/or CV risk factors	16♂, 4♀	CD34^{+}CD133^{+}VEGFR2^{+} EPCs	2.9 fold increase	Steiner *et al*., 2005

CAD =Coronary Artery Disease, CV =Cardiovascular, EPC=Endothelial Progenitor Cell, PAOD =Peripheral Arterial Occlusive Disease,♂ = male subjects, ♀ = female subjects.

Table 2.6. Summary of Exercise Training Studies and Endothelial Progenitor Cells (continued).

Form of Exercise	Subject Characteristics	♂/♀	EPC Definition	Result	Reference
8 weeks cycling aerobic endurance training	8 sedentary old men	8♂	$CD34^+VEGFR2^+$ EPCs	No change	Thijssen *et al*., 2006
23 week aerobic exercise training	10 middle aged and 10 older men	20♂	EPC EC-CFU	Increase in EPC EC-CFU and EPC migration ability	Hoetzer *et al*., 2007
6 months walking exercise training	30 dialysis patients	10♂, 4♀	$CD34^+CD133^+VEGFR2^+$ EPC EC-CFU	No change in EPC number EPC EC-CFU increased	Manfredini *et al*., 2009
6 months supervised aerobic exercise training	20 patients with PAD	13♂, 7♀	$CD34^+CD133^+VEGFR2^+$ EPC EC-CFU	Increase EPC number Increase in EC-CFU and migration ability	Schlager *et al*., 2011

EC-CFU = Endothelial Cell-Colony Forming Units, EPC = Endothelial Progenitor Cell, PAD = Peripheral Arterial Disease,

♂=male subjects, ♀ = female subjects.

The majority of these studies have investigated the effects of exercise on cardiovascular disease patients, and found that although circulating EPC levels increase, the response is attenuated in cardiovascular disease patients compared to healthy controls (Sandri *et al*., 2011; Van Craenenbroeck *et al*., 2011). This indicates a reduced exercise-induced EPC-mediated angiogenic response in disease patients compared to healthy populations. Van Craenenbroeck *et al* (2008) also observed an increase in EPCs, which correlated to subject's fitness levels, indicating that there is an increased response in physically fit individuals.

An increase in EPC functional ability (either measured by CFU or migratory capacity) has been observed as a result of acute (Laufs *et al*., 2005) and chronic exercise training (Manfredini *et al*., 2009; Schlager *et al*., 2011).

The evidence suggests that the response of EPCs to aerobic exercise to be either/both intensity-dependent (Bonsignore *et al*., 2010) or/and exercise duration-dependent (Laufs *et al*., 2005; Möbius Winkler *et al*., 2009). However, the intensity-dependent response was only seen in $CD133^{+}VE\text{-}cadherin^{+}$ EPCs, and not in $CD34^{+}VEGFR2^{+}$ EPCs.

These acute exercise-mediated increases in EPC number were accompanied by increases in plasma VEGF (Adams *et al*., 2004; Sandri *et al*., 2011) and IL-6 (Goussetis *et al*., 2009; Möbius-Winkler *et al*., 2009). The increase in EPCs has also been shown to be accompanied by increases in C-reactive protein (CRP), IL-6, IL-8, VCAM-1, ICAM-1 and thrombomodulin in one study (Goussetis *et al*., 2009), indicative of acute inflammation. Increased exercise-induced shear stress may also increase circulating EPC numbers through detaching any EPCs which are adhering to the EC wall (Rehman *et al*., 2004) or detach from stromal cells (Aicher *et al*., 2005).

In summary the majority of studies involving aerobic exercise have shown increases in circulating EPC number. However it has yet to be elucidated what duration, intensity and the environmental conditions by which exercise causes the greatest improvements in EPC number or function, both acutely and chronically.

Chapter 3

Methodology

3.0 Methodology

3.1 Study 1: 'The Influence of Acute High Volume Low Intensity Resistance Exercise Bout on Circulating Endothelial Progenitor Cells and Endothelial-Derived Microparticles in Trained Men'

3.1.1 Study Overview

The purpose of the study was to determine the influence of a high volume low intensity resistance exercise bout on circulating endothelial progenitor cell (EPC) and endothelial microparticle (EMP) numbers. Thirteen resistance-trained men performed 3 sets of resistance exercise. Blood samples were taken pre-exercise and 10 minutes, 2 hours and 24 hours post-exercise. These were analysed for EPCs, EMPs along with growth factors that might explain changes in EPCs.

3.1.2 Subjects

Thirteen trained male subjects participated in a high volume low intensity resistance exercise (RE) protocol. Subjects were asked to complete a physiological screening questionnaire (appendix B) in order to ensure they had no cardiovascular risk factors prior to the study. Written informed consent (appendix C) was obtained from each subject. The study was approved by the Waterford Institute of Technology Ethics Committee.

3.1.3 Baseline Measures

During the subjects' first visit they were measured for height and weight, as well as resting blood pressure. A dual x-ray absorptiometry (DXA) scan (Nordland, USA) was performed to attain percentage body fat (%BF) and percentage of lean mass (%LM). Subjects were also assessed for their 15 repetition maximum (RM) for the following exercises: bench press, leg press, seated press, lat pulldown, leg extension, leg curl, and tricep pushdown.

Table 3.1. Baseline Characteristics.

Characteristic	RE Group (n=13)
Age (years)	22 ± 2
Height (cm)	178.2 ± 7.0
Body Mass (kg)	82.4 ± 11.3
BMI (kg/m^2)	25.9 ± 2.3
Body Fat (%)	15.3 ± 5.0
Lean Mass (%)	81.0 ± 4.8

Values shown are mean ± SD

3.1.4 Exercise Bout and Dietary Control

One week later, subjects performed the RE bout in the morning after an overnight fast. The exercise bout consisted of 3 circuits of leg press, seated chest press, leg curl, lat pulldown, knee extension, and tricep pushdown for 15 repetitions at 15RM in that sequence. All exercises were performed on machine weights in order to eliminate

technical issues with free weights. Blood lactate (BLa) was measured from finger tip capillary blood sample taken pre-exercise, in between each circuit and post-exercise (LactatePro, H/P/Cosmos, Germany). Heart rate (HR) was measured pre-exercise, and in between each circuit as well as after the last circuit with the subject wearing a HR monitor (Polar, Finland). The subjects were asked to record their diet on the day prior to the RE bout, and repeat this after the exercise in the lead up to a 24 hours-post blood sample.

3.1.5 Blood Collection and Processing

Peripheral blood samples were used to measure circulating EPCs, cell-derived MPs, soluble factors and EMP-bound E-selectin. Peripheral blood (9mL, 1 ethylene diamine tetraacetic acid [EDTA], 1 sodium citrate, and 1 serum vacutainers BD Biosciences, USA) was collected at several time points (pre-, 10 minutes post-, 2 hours post-, and 24 hours post-exercise) using a 21-gauge needle (BD Biosciences, USA). An initial 3mL of blood was discarded to avoid local MP release due to the insertion of the needle. There was minimal use of the tourniquet to avoid further endothelial damage or cell activation.

The EDTA tube was mixed gently, top removed and used to obtain a complete blood cell count using an automated haematology analyser (AcT Diff, Beckman Coulter, USA). Then 200µL was pipetted into a flow cytometry tube for EPC quantification, and another 200µL pipetted into a second tube which served as the negative control tube. The remainder was centrifuged at 1500g for 15 minutes at room temperature in a Sigma 2-16 centrifuge (Sigma Centrifuges, SciQuip, Shropshire, UK).

The sodium citrate vacutainer was allowed to stand for 30 minutes at room temperature prior to processing. It was then centrifuged at 1500g for 15 minutes at room temperature

in a Sigma 2-16 centrifuge (Sigma Centrifuges, SciQuip, Shropshire, UK). Platelet-poor plasma (PPP) was collected from the sodium citrate vacutainer (leaving 1cm above buffy coat) and centrifuged again at 13000g for 2 minutes at room temperature. The platelet-free plasma (PFP) was collected (leaving 500µl remaining in eppendorf) and 350µL aliquoted into eppendorfs and samples were frozen at -80ºC. This double centrifugation process is recommended and serves to remove large cells without further cell activation using a gentle first spin and a subsequent more vigorous spin to remove most platelets.

The serum vacutainer was left to stand for 30 minutes at room temperature prior to processing. Then the vacutainer was centrifuged at 1500g for 15 minutes at room temperature. The supernatant from the serum vacutainer was collected (leaving 1cm above buffy coat) and 350µL of sample aliquoted into eppendorfs and frozen at -80°C.

These steps were performed for each timepoint that peripheral blood was collected.

3.1.6 Measurement of Circulating Endothelial Progenitor Cells

For flow cytometric analysis of EPCs, whole blood from EDTA vacutainers was used. Briefly, 200µL of EDTA whole blood was incubated with 50µL of Fc receptor blocker (Beckman Culter, Inc. USA) for 15 minutes in the dark in order to reduce non-specific binding when the primary antibodies were added. Subsequently the sample was incubated with 20µL CD34-PECy7 (Beckman Coulter, Inc.USA), 20µL vascular endothelial growth factor receptor 2 (VEGFR2)-PE (R&D Systems Inc. USA), and 20µL CD45-FITC (Beckman Coulter, Inc. USA) for 30 minutes in the dark. Subsequently 2mL of Pharm Lyse™ (BD Biosciences, USA) was added in order to lyse erythrocytes prior to flow cytometric analysis. The sample was incubated for a further

20 minutes. The sample was run through the flow cytometer (FC500, Beckman Coulter, USA) for 30 minutes. A negative isotype matched control was run prior to analysis of a positive sample to distinguish progenitor cells positive and negative for VEGFR2. This sample was processed identically to the positive sample but 20µL isotype for PE (R&D Systems Inc. USA) was added instead VEGFR2.

EPCs were quantified using a modified version of the ISHAGE protocol for progenitor cells (Sutherland *et al.*, 1996) with the addition of VEGFR2 marker to identify progenitors of endothelial origin. Thus EPCs were identified as $CD34^{+}VEGFR2^{+}CD45^{dim}$. Total EPC events were converted to cells/mL using the dual platform method in conjunction with the haematology analyser determined leukocyte count.

($CD34^{+}VEGFR2^{+}CD45^{dim}$ counts x (WBC count/CD45+ count)) x 1000

WBC= White Blood Cell

The EPC gating strategy is documented in appendix D.

3.1.7 Measurement of Cell-Derived Microparticles

For flow cytometric analysis of MPs, frozen PFP samples were thawed at room temperature. Samples were analysed to enumerate cell-derived microparticles <1.0µm in size. Briefly 30µL of sample was incubated with either 5µL CD144 (Beckman Coulter, Inc. USA), 5µL CD62E-PE-Cy5 (BD Biosciences/Pharmigen™, USA) to determine EMP number, 5µL CD235a (Beckman Coulter, Inc. USA) to determine Erythrocyte MP (ErMP) number, or 10µL CD41-PE (PMP marker; Biocytex, France) with 10µL AnV-FITC (Beckman Coulter, Inc. USA) to determine platelet MP (PMP)

number, for 30 minutes in the dark. Either 500µL of phosphate-buffered solution (PBS) (EMP and ErMP analysis) or 500µL annexin V binding buffer (for PMP analysis; contains calcium and necessary for annexin V binding) was added immediately prior to flow cytometric analysis which consisted of a 2 minute analysis on a low analysis speed. The 1.0µm gate was established using sizing beads (Megamix Beads, Biocytex, France). The mean speed on the low analysis setting was determined by the addition of SPHERO™ Accucount Particles (Spherotech, Inc., USA) which resulted in a flow speed of 9.5µL·min^{-1} during low speed analysis by the flow cytometer.

A negative control sample was prepared to distinguish CD144 positive and negative events using 5µL of IgG1 (Mouse)-PE isotype control (Beckman Coulter, Inc. USA) as CD144^{+} EMP are only weakly positive for the antigen. For all other MP analyses, a clear separation was evident between events positive and negative for the marker(s) of interest negating the need for isotype control samples. PMP formed a distinct cluster of events with strong dual positivity for CD41 and annexin V (appendix E).

Details regarding analyses for CD62E^{+} EMPs, CD144^{+} EMPs, CD41^{+}annexin V^{+} PMPs, CD235a^{+} ErMPs are detailed in appendix E.

3.1.8 Measurement of Microparticle-Bound and Soluble E-Selectin

Sodium citrate plasma samples prepared using the double centrifugation protocol were analysed for the presence of MP-bound and soluble CD62E (E-Selectin) in a sandwich immunoassay that uses electrochemiluminescence detection (Mesoscale Discovery, Maryland, USA). MP-bound E-selectin determined using an immunoassay can reinforce flow cytometry results. MP-bound E-selectin enters circulation as part of an MP during MP budding and release. Soluble E-selectin results from the cleavage of the

transendothelial domain of the molecule while still attached to parent ECs, resulting in the release of the remainder of the molecule into the circulation. Increased levels of soluble E-selectin can also represent increased expression of CD62E on EC membranes.

Briefly, frozen PFP was thawed at room temperature. Subsequently, 350µL PFP samples were spun at 19000g for 30 minutes at room temperature in order to separate soluble samples of E-selectin and MP-bound samples of E-selectin. The top 300µL supernatant was removed and pipette into a separate eppendorf. This sample was to be used for analysis of the soluble E-selectin. The pellet was resuspended in 300µL of PBS and re-spun at 19000g for 30 minutes. The supernatant (310µL) was again removed, this time disposed of. The remaining 40µL pellet and the supernatant from the first spin was used for the subsequent analysis for MP-bound E-selectin by elechtrochemiluminescence assay.

The protocols for the detection of EMP-bound and soluble E-selectin are detailed in appendices F and G respectively.

3.1.9 Measurement of Soluble Factors and Cytokines

Serum vascular endothelial growth factor (VEGF) and granulocyte-colony stimulating factor (G-CSF) were analysed from serum samples using an immunoassay that uses electrochemiluminescence detection (Mesoscale Discovery, Maryland, USA). Details regarding the procedures for each are documented in appendices H and I respectively.

3.2 Study 2: 'The Influence of Acute Aerobic Exercise Bout on Circulating Cell-Derived Microparticles in Trained Men'

3.2.1 Study Overview

The purpose of the study was to determine the influence of an aerobic exercise bout on circulating EMP numbers. Nine aerobically-trained men performed 45 minutes treadmill running at 75% maximal aerobic capacity ($\dot{V}O_2$max). Blood samples were taken pre-exercise and immediately, 1 hour and 4 hours post-exercise. These were analysed for EMPs, PMPs and ErMPs.

3.2.2 Subjects

Nine trained male subjects participated in an aerobic exercise (AE) exercise protocol. Subjects were asked to complete a physiological screening questionnaire (appendix B) in order to ensure they had no cardiovascular risk factors prior to the study. Written informed consent (appendix J) was obtained from each subject. The study was approved by the Waterford Institute of Technology Ethics Committee.

3.2.3 Baseline Measures

During the subjects' first visit they were measured for height and weight, as well as resting blood pressure. A DXA scan (Nordland, USA) was performed to attain %BF and %LM. Subjects were also assessed for their $\dot{V}O_2$max through an incremental treadmill running protocol to volitional exhaustion on a treadmill ergometer. HR was measured at rest and during the exercise test using a HR monitor (Polar, Finland) and BLa

(LactatePro, H/P/Cosmos, Germany) was also measured at rest and throughout the maximal exercise test.

Table 3.2. Baseline Characteristics.

Characteristic	AE Group (n=9)
Age (years)	23 ± 2
Height (cm)	1.82 ± 0.1
Body Mass (kg)	82.2 ± 10.0
BMI (kg/m^2)	24.9 ± 2.3
Body Fat (%)	16.7 ± 5.7
Lean Mass (%)	79.6 ± 6.9
$\dot{V}O_2$max ($ml \cdot kg \cdot min^{-1}$)	60.5 ± 7.0

Values shown are mean ± SD

3.2.4 Exercise Bout

Subjects returned one week after initial baseline testing. Subjects performed 45 minutes running exercise on a treadmill ergometer at 75% $\dot{V}O_2$max. This was undertaken 4 hours after a set breakfast (35g commercially available cereal [Frosties, Kellogs®, Michigan, USA], 35mL low fat milk, followed by 3 slices of dry white bread toast). BLa was measured pre- and post-exercise using a capillary blood sample from a fingertip.

3.2.5 Blood Collection and Processing

Peripheral blood samples were collected to measure cell-derived MPs. Peripheral blood (1 EDTA and 1 sodium citrate vacutainers, BD Biosciences, USA) was collected at several time points (pre-, immediately post-, 1 hour post- and 4 hours post-exercise) using a 21-gauge needle (BD Biosciences, USA). The first 3mL of blood was discarded to avoid local MP release due to the insertion of the needle. There was minimal use of the tourniquet to avoid further endothelial damage or cell activation.

Blood processing and analysis was carried out as previously descried. Whole blood EDTA collected was analyzed for haematology counts (AcT Diff, Beckman-Coulter, USA).

3.2.6 Measurement of Cell-Derived Microparticles

For flow cytometric analysis of MPs (EMP, ErMP, PMP), frozen PFP was thawed at room temperature. Sample preparation and incubation with relevant mAbs are detailed in previous substudy. MPs were detected by flow cytometry by gating events <1.0μm in size. Details regarding analyses for $CD62E^+$, $CD144^+$ EMPs, $CD41^+$annexin V^+ PMPs, $CD235a^+$ ErMPs are detailed in appendix E.

3.3 Statistical Analysis

In both the RE and AE studies, the data were normally distributed. The significance of changes over timepoints were determined using a one-way repeated measures Analysis of Variance (ANOVA). Significant F ratios were followed up by the Least Significant Differences post-hoc procedure. Associations between variables and changes in variables were determined using Pearsons correlations. Significance was set at $p<0.05$. Statistical analysis was performed using SPSS version 17 software (SPSS®, IBM®, USA).

The intraclass correlations determined in our lab for repeated measures of EPC and EMP were 0.76 and 0.84 respectively. Based on these correlations, the studies were powered to detect changes in EPC and EMP of moderate effect size (Cohen's $f = 0.25$) with $n= 12$ and $n= 10$ respectively, assuming $\alpha=0.05$ and $1-\beta = 0.80$ (desired power of 80%). Effect sizes of 0.10, 0.25 and 0.40 can be regarded as small, medium and large effect sizes based on the Cohen's f effect size statistic used in analysis of variance research designs (Cohen, 1988). This statistic expresses effect size in standard deviation units for more than two groups. Sample size calculations were undertaken using G*Power 3.1.2 statistical software (Erdfelder *et al*., 1996). In the results section, the effect size for each one way repeated measures ANOVA is reported. This was calculated using a combination of SPSS version 17.0 and G*Power 3.1.2.

Chapter 4

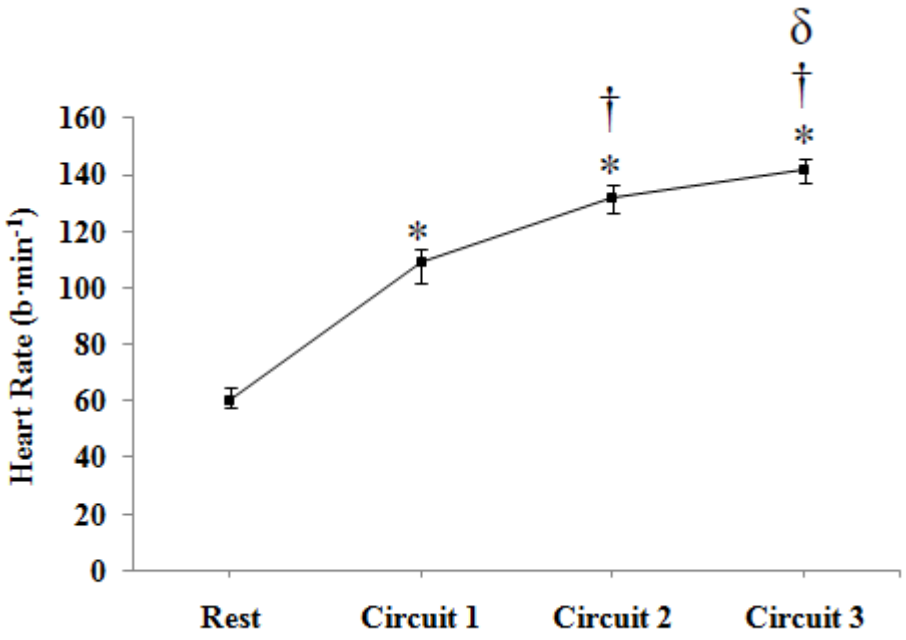
Presentation of Results

4.0 Presentation of Results

4.1 Study 1: 'The Influence of Acute High Volume Low Intensity Resistance Exercise on Circulating Endothelial Progenitor Cells and Cell-Derived Microparticles in Trained Men'

4.1.1 Physiological Responses to Resistance Exercise Bout

Figure 4.1. Heart Rate Response to Resistance Exercise in Trained Men (n=13).

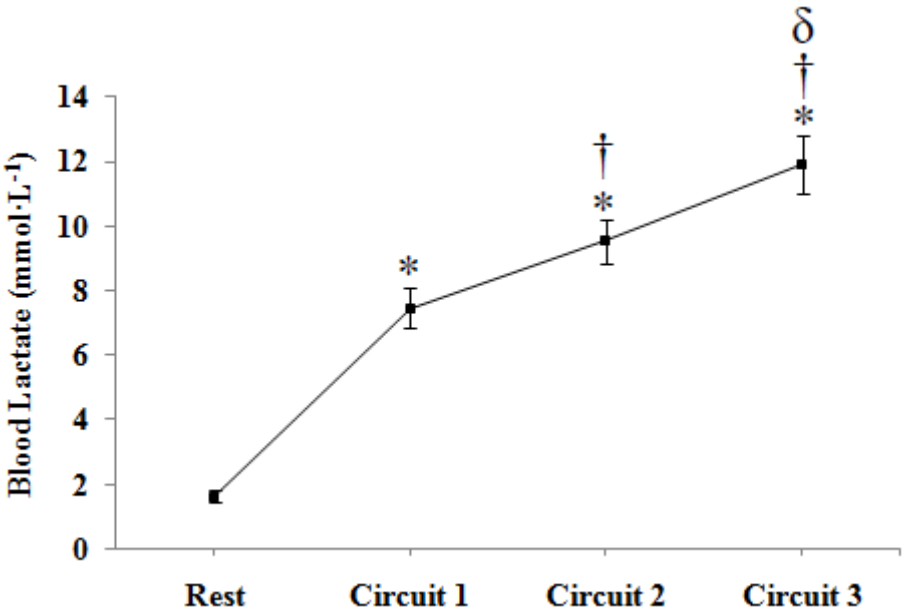


Values shown are mean ± SE

** p<0.05 significant different from pre-exercise levels, † p<0.05 significant different from circuit 1, δ p <0.05 significant different from circuit 2. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

The resistance exercise bout produced a significant increase in heart rate (HR) from pre-exercise to the end of circuit 1 (60 ± 3 to 109 ± 7 b·min^{-1}, $p<0.001$), circuit 2 (60 ± 3 to 132 ± 6 b·min^{-1}, $p<0.001$) and circuit 3 (60 ± 3 to 142 ± 5 b·min^{-1}, $p<0.001$). There was a significant increase from the end of circuit 1 to the end of circuit 2 (109 ± 7 to 132 ± 6 b·min^{-1}, $p<0.001$) and to the end of circuit 3 (109 ± 7 to 142 ± 5 b·min^{-1}, $p<0.005$), and from the end of circuit 2 to the end of circuit 3 (132 ± 6 to 142 ± 5 b·min^{-1}, $p<0.001$).

Figure 4.2. Levels of Blood Lactate Pre- and Post- Resistance Exercise in Trained Men (n=13).



Values shown are mean ± SE

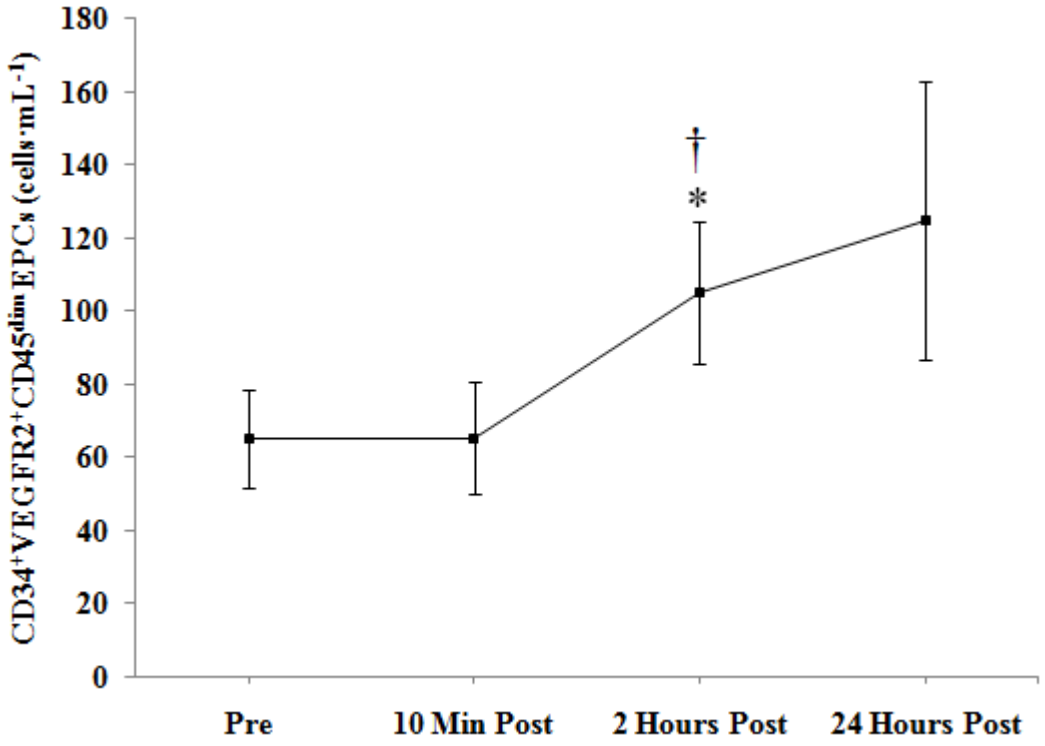
** p<0.05 significant difference from pre-exercise levels, † p<0.05 significant difference from end of circuit 1, δ p<0.05 significant difference from end of circuit 2. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

The resistance exercise bout also produced a significant increase in blood lactate (BLa) levels from pre-exercise to end of circuit 1 (1.5 ± 0.2 to 7.5 ± 0.6 mmol·L^{-1} p<0.001), circuit 2 (1.5 ± 0.2 to 9.5 ± 0.7 mmol·L^{-1} p<0.001), and end of circuit 3 (1.5 ± 0.2 to 11.9 ± 0.9 mmol·L^{-1} p<0.001). There was an increase from end of circuit 1 to end of circuit 2 (7.5 ± 0.6 to 9.5 ± 0.7 mmol·L^{-1} p<0.001) and end of circuit 3 (7.5 ± 0.2 to 11.9

± 0.9 mmol·L^{-1} $p<0.001$), as well as an increase from the end of circuit 2 to the end of circuit 3 (9.5 ± 0.7 to 11.9 ± 0.9 mmol·L^{-1} $p<0.001$).

4.1.2 Endothelial Progenitor Cells

Figure 4.3. Endothelial Progenitor Cell counts (defined as $CD34^{+}VEGFR2^{+}CD45^{dim}$) Pre- and Post- Resistance Exercise in Trained Men (n=13).



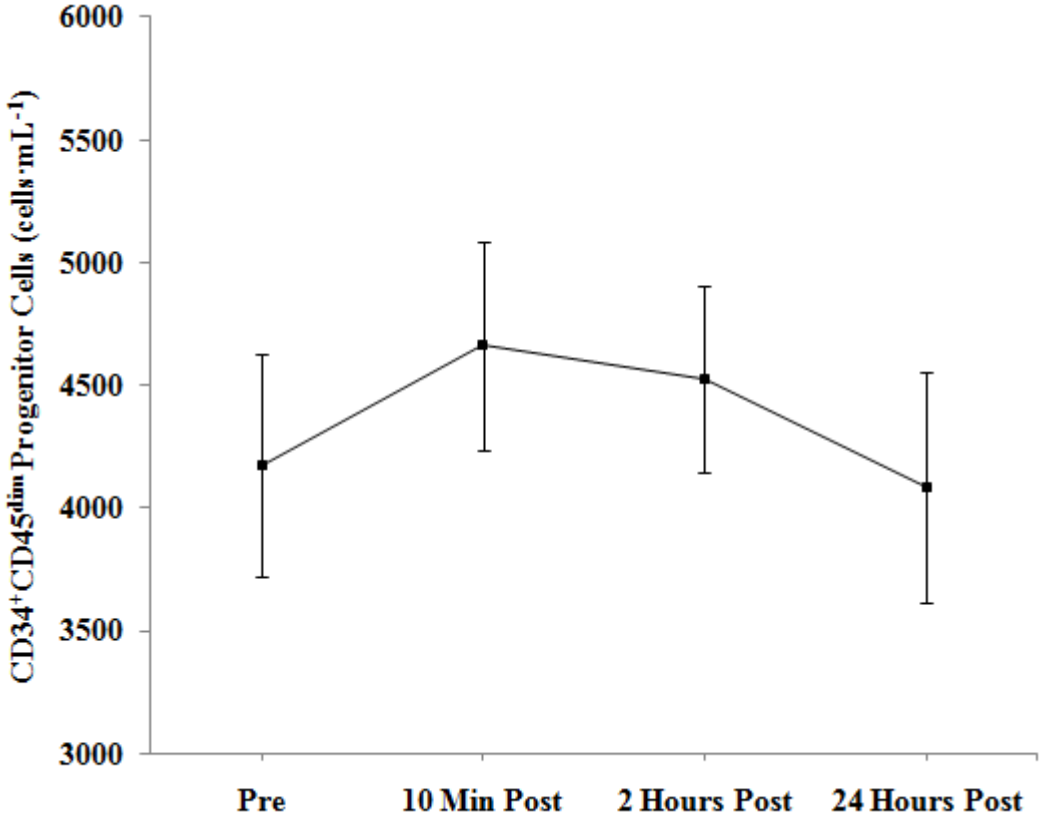
Values shown are mean ± SE

**p<0.05 significant difference from pre-exercise levels, † p<0.05 significant difference from 10 minutes post-exercise levels. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There was a significant increase in endothelial progenitor cell (EPC) counts ($CD34^{+}VEGFR2^{+}CD45^{dim}$) as a result of the resistance exercise bout ($p<0.05$, Cohen's f

effect size=0.49). EPCs increased from pre- to 2 hours post-exercise (65 ± 13 to 105 ±19 cells·mL^{-1} $p<0.05$) as well as from 10 minutes post-exercise to 2 hours post-exercise (65 ± 15 to 105 ± 19 cells·mL^{-1}, $p<0.005$) as a result of this particular bout of exercise.

Figure 4.4. Total Progenitor Cell Counts (defined as $CD34^{+}CD45^{dim}$) Pre- and Post- Resistance Exercise in Trained Men (n=13).

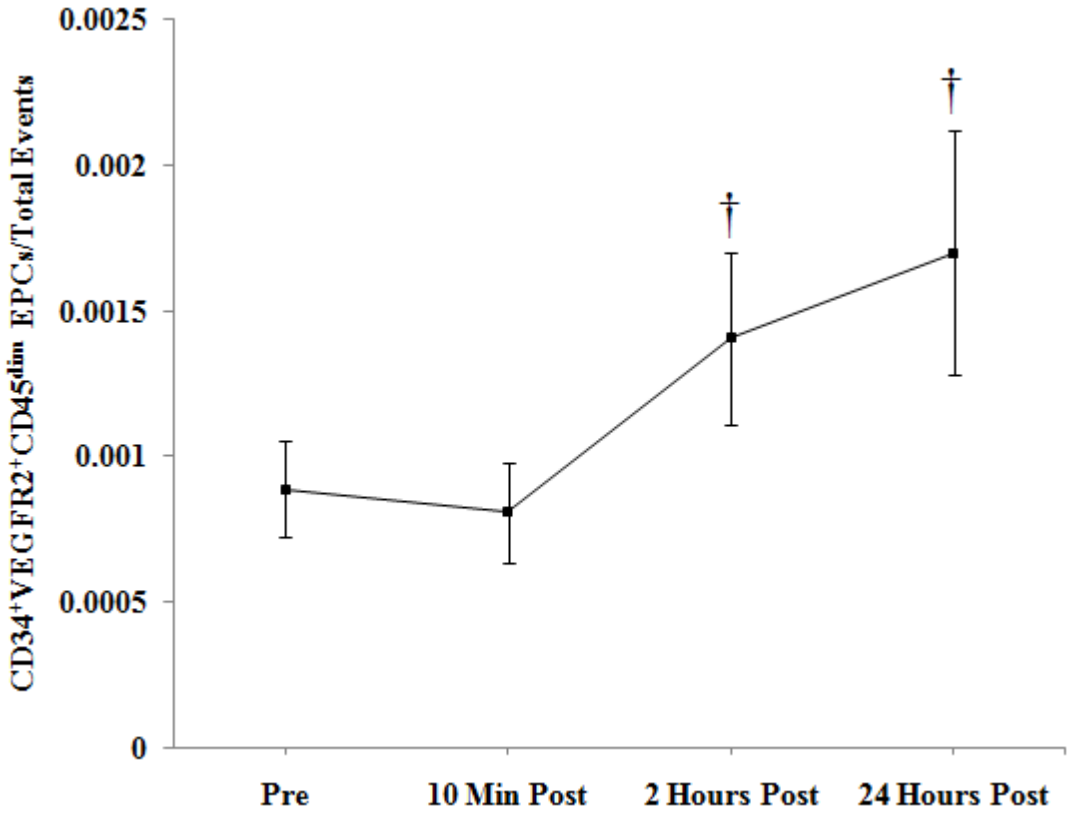


Values shown are mean ± SE.

Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.

There was no significant change in circulating progenitor cells (defined as $CD34^{+}CD45^{dim}$) as a result of the resistance exercise bout ($p=0.32$, Cohen's f effect size=0.32).

Figure 4.5. Endothelial Progenitor Cells (defined as $CD34^{+}VEGFR2^{+}CD45^{dim}$) as a Percentage of Total Events ($CD45^{+}$) Pre- and Post- Resistance Exercise in Trained Men (n=13).



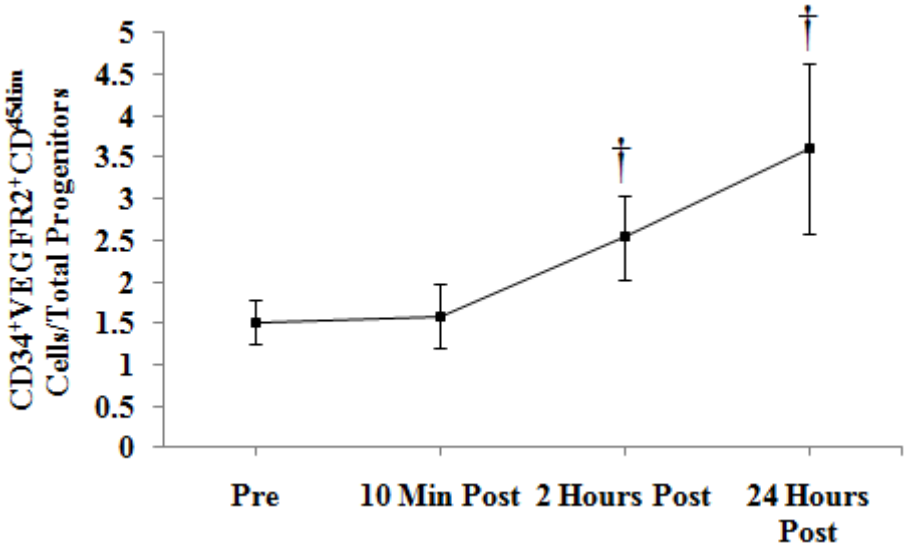
Values shown are mean ± SE

† *p<0.05 significant difference from 10 minutes post-exercise levels. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There was a significant increase in EPCs when expressed as a percentage of total $CD45^{+}$ events as a consequence of the resistance exercise bout ($p<0.05$, Cohen's f effect size=0.58). There was a significant increase from 10 minutes post-exercise to 2 hours

post-exercise (0.000809 ± 0.000175 to 0.001405 ± 0.000296%, $p<0.05$) and 24 hours post-exercise (0.000809 ± 0.000175 to 0.001701 ± 0.000423%, $p<0.05$).

Figure 4.6. Endothelial Progenitor Cells (defined as $CD34^{+}VEGFR2^{+}CD45^{dim}$) as a Percentage of Total $CD34^{+}CD45^{dim}$ cells Pre- and Post- Resistance Exercise in Trained Men (n=13).



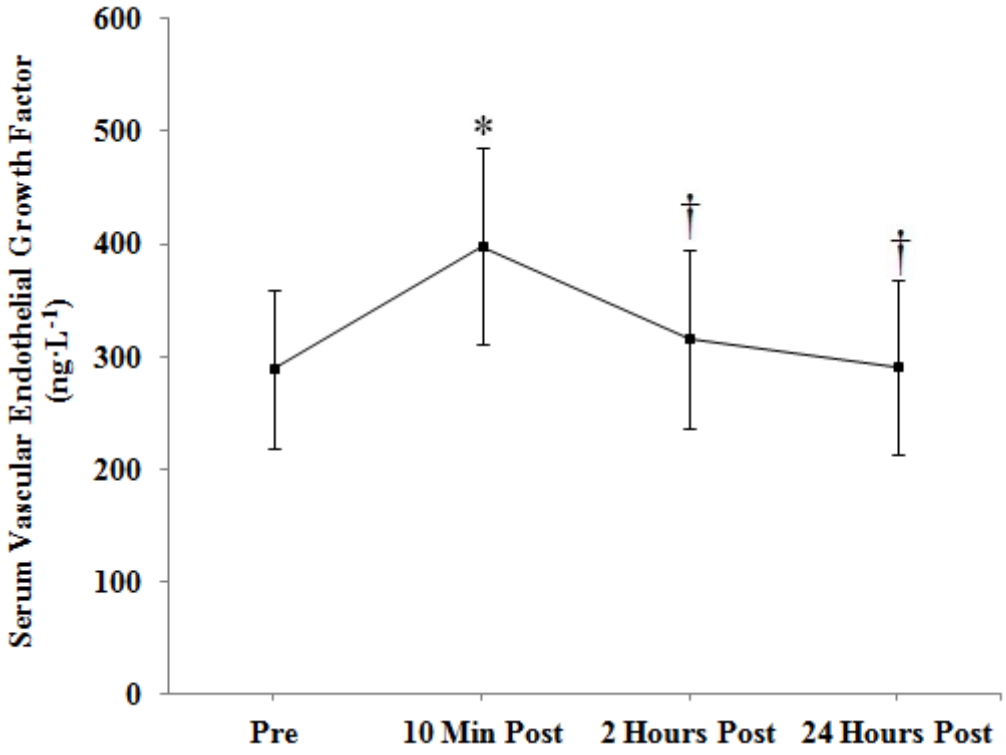
Values shown are mean ± SE

† p<0.05 significant different from 10 minutes post-exercise levels. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.

There was a significant increase in the percentage of progenitor cells (defined as $CD34^{+}CD45^{dim}$) expressing VEGFR2 as a result of the resistance exercise bout ($p<0.05$, Cohen's f effect size=0.57). There were significant increases from 10 minutes post-exercise to 2 hours post-exercise (1.59 ± 0.39 to 2.54 ± 0.50%, $p<0.01$) and to 24 hours post-exercise (1.59 ± 0.39 to 3.60 ± 1.03%, $p<0.05$). There was a change approaching significance from pre-exercise to 10 minutes post-exercise ($p=0.071$) and to 2 hours post-exercise ($p=0.070$).

4.1.3 Soluble Factors

Figure 4.7. Serum Vascular Endothelial Growth Factor (VEGF) Pre- and Post-Resistance Exercise in Trained Men (n=13).



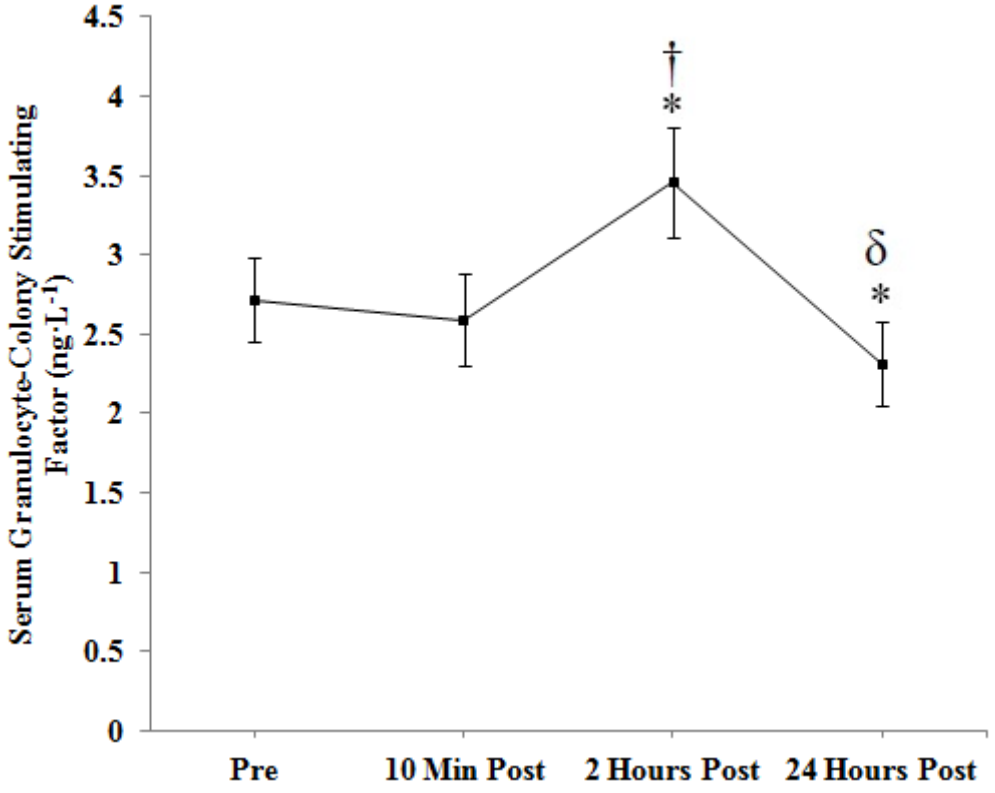
Values shown are mean ± SE

**p<0.05 significant difference from pre-exercise levels, † p<0.05 significant difference from 10 minutes post-exercise levels. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There were significant changes in serum VEGF levels as a result of the resistance exercise bout ($p<0.05$, Cohen's f effect size=0.68). There was a significant increase from pre- to 10 minutes post-exercise (289 ± 70 to 398 ± 87 $ng \cdot L^{-1}$, $p<0.05$), and a

decrease from 10 minutes post-exercise to both 2 hours post- (398 ± 87 to 316 ± 79 $ng \cdot L^{-1}$, $p<0.05$) and 24 hours post-exercise (398 ± 87 to 291 ± 78 $ng \cdot L^{-1}$, $p<0.05$).

Figure 4.8. Serum Granulocyte-Colony Stimulating Factor (G-CSF) Pre- and Post-Resistance Exercise in Trained Men (n=13).



Values shown are mean ± SE

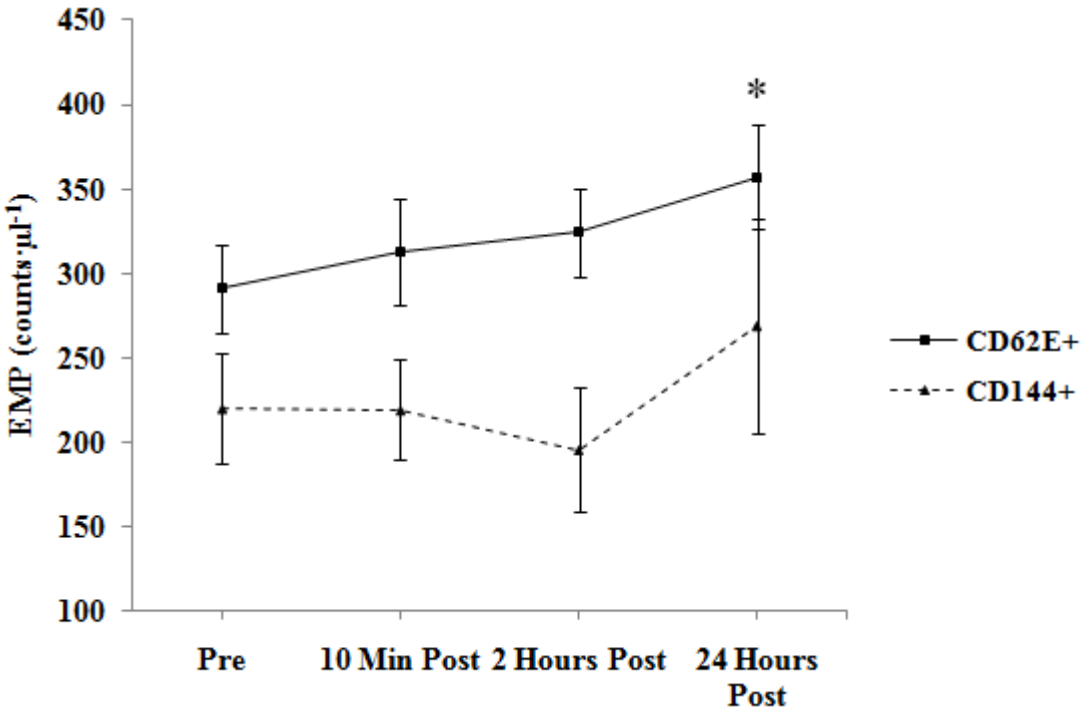
** p<0.05 significant difference from pre-exercise levels, † p<0.05 significant difference from 10 minutes post-exercise levels, δ p<0.05 significant difference from 2 hours post-exercise levels. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There were significant changes in serum G-CSF as a consequence of the resistance exercise bout ($p<0.05$, Cohen's f effect size=1.21). A significant increase was observed from pre- to 2 hours post-exercise (2.7 ± 0.3 to 3.5 ± 0.4 ng·L^{-1}, $p<0.05$) and from 10 minutes post-exercise to 2 hours post-exercise (2.6 ± 0.3 to 3.5 ± 0.4ng·L^{-1}, $p<0.05$) while there was a decrease at 24 hours post-exercise which was significantly reduced

from baseline (2.7 ± 0.3 down to 2.3 ± 0.3 $ng \cdot L^{-1}$, $p<0.001$) and from 2 hours post-exercise (3.5 ± 0.4 down to 2.3 ± 0.3 $ng \cdot L^{-1}$, $p<0.001$).

4.1.4 Cell-Derived Microparticles

Figure 4.9. Circulating Endothelial Microparticle Counts Pre- and Post-Resistance Exercise in Trained Men (n=13).



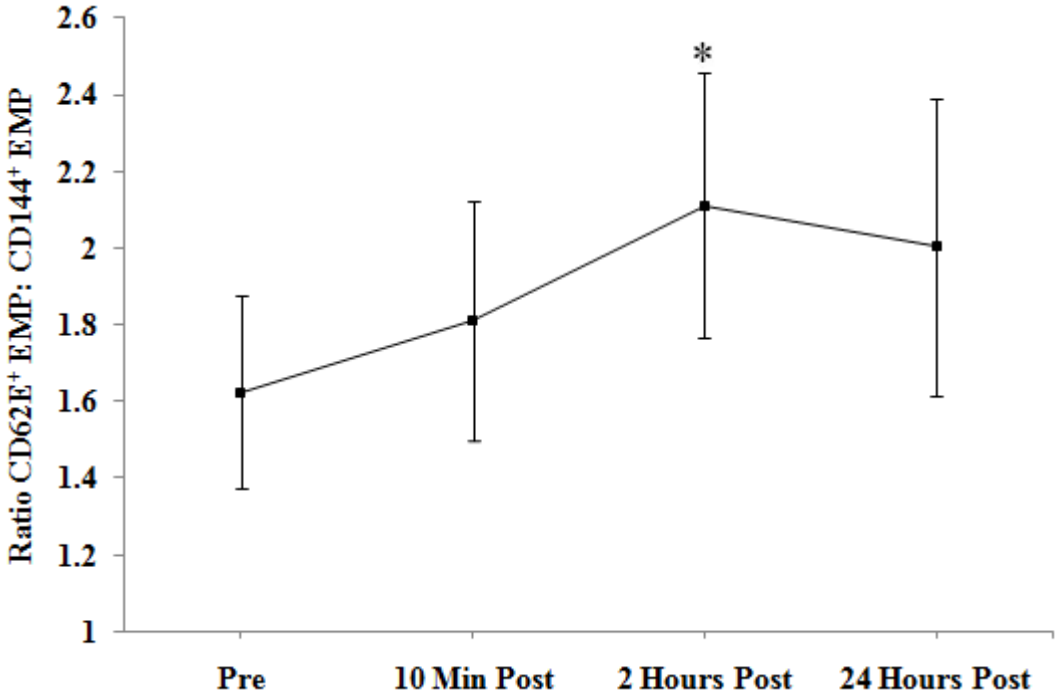
Values shown are mean ± SE

**p<0.05 significant difference from pre-exercise levels. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There was a significant increase in circulating $CD62E^+$ endothelial microparticles (EMP) as a result of the resistance exercise bout ($p<0.05$, Cohen's f effect size=0.51). A significant increase was found from pre- to 24 hours post-exercise (291 ± 26 to 357 ± 31 counts·μL^{-1}, $p<0.05$). There was a change approaching significance in $CD62E^+$ EMP

counts between pre- and 10 minutes post-exercise ($p=0.072$), as well as between 2 hours post-exercise and 24 hours-post exercise ($p=0.062$). There was no change in $CD144^{+}$ EMP levels as a result of the exercise bout ($p=0.13$, Cohen's f effect size=0.24).

Figure 4.10. Ratio of $CD62E^+$ Endothelial Microparticles: $CD144^+$ Endothelial Microparticles Pre- and Post- Resistance Exercise in Trained Men (n=13).

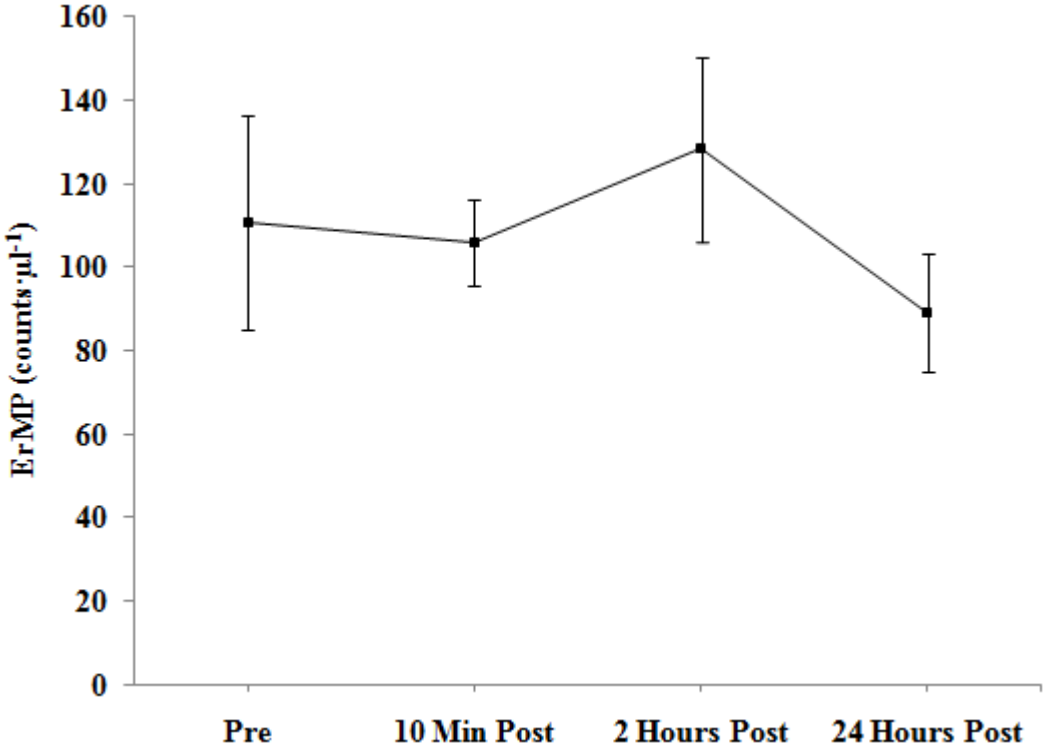


Values shown are mean ± SE.

** p<0.05 significant difference from pre-exercise levels. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There was a significant increase in the ratio of $CD62E^+$ EMP to $CD144^+$ EMP as a result of the resistance exercise bout ($p<0.05$, Cohen's f effect size=0.45). There was a significant increase from pre- to 2 hours post-exercise (1.62 ± 0.25 to 2.11 ± 0.35, $p<0.05$). There was a change approaching significance from pre- to 24 hours post-exercise ($p=0.069$).

Figure 4.11. Circulating Erythrocyte Microparticle Counts Pre- and Post-Resistance Exercise in Trained Men (n=13).

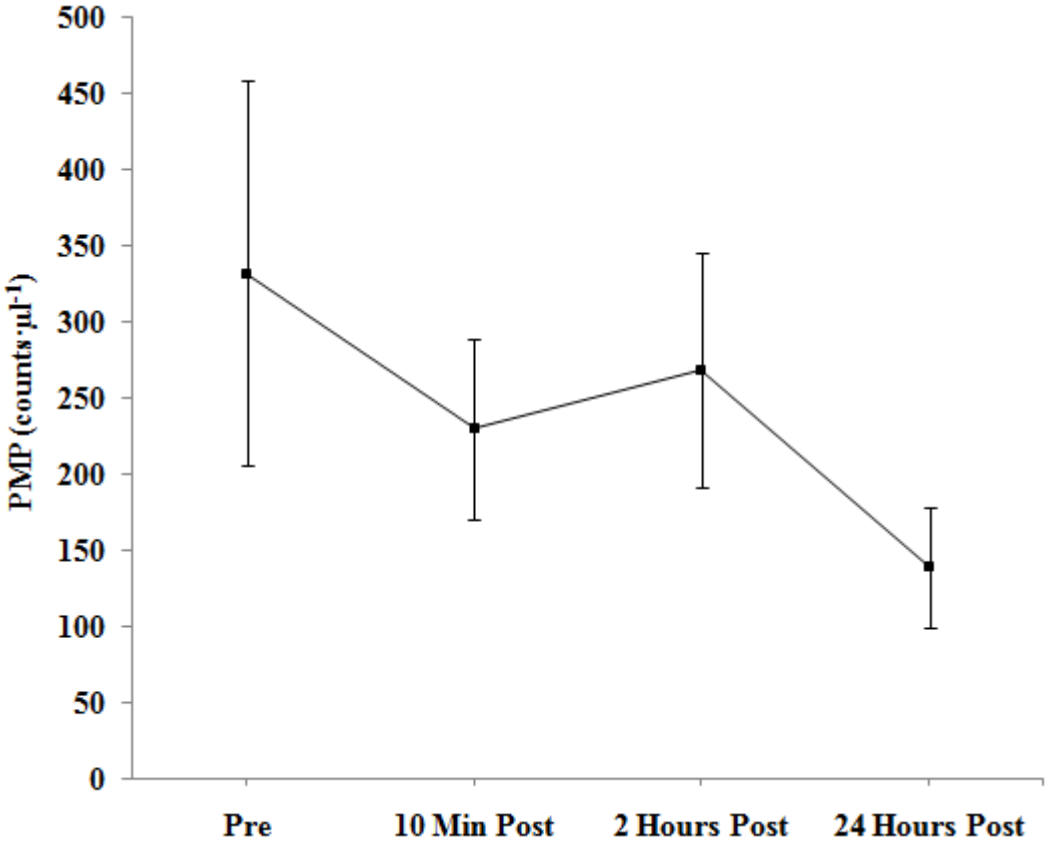


Values shown are mean ± SE.

Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.

There was no significant changes in circulating erythrocyte microparticle (ErMP) count as a result of the resistance exercise bout (p=0.57, Cohen's f effect size=0.41).

Figure 4.12. Circulating Platelet Microparticle Counts Pre- and Post- Resistance Exercise in Trained Men (n=13).



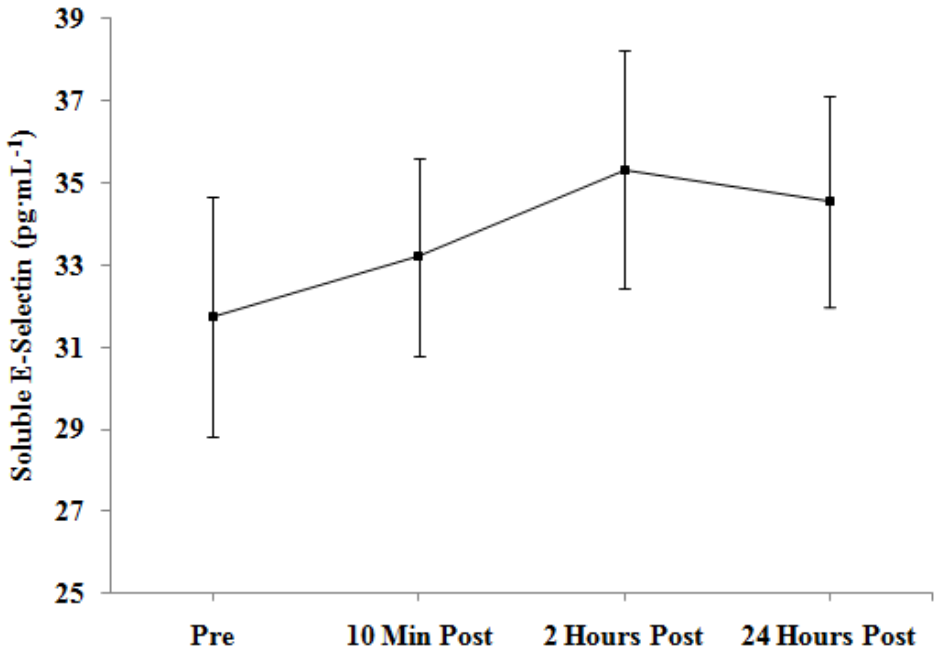
Values shown are mean ± SE.

Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.

There were no significant changes in circulating platelet microparticles (PMP) as a result of resistance exercise (p=0.13, Cohen's f effect size=0.24).

4.1.5 Markers of Endothelial Activation

4.13. Soluble E-Selectin Pre- and Post- Resistance Exercise in Trained Men (n=8).

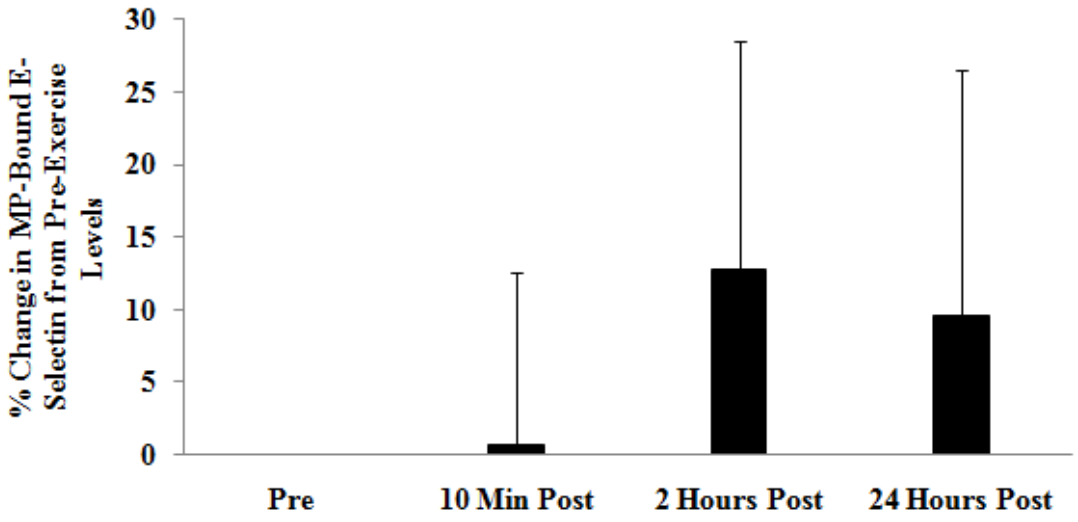


Values shown are mean ± SE.

Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.

There was an increase in soluble E-selectin that approached significance (p=0.12, Cohen's f effect size=0.55) as a result of the resistance exercise bout.

Figure 4.14. Change from Pre-Exercise in Microparticle-Bound E-selectin Post-Resistance Exercise in Trained Men (n=12).



Values shown are mean ± SE

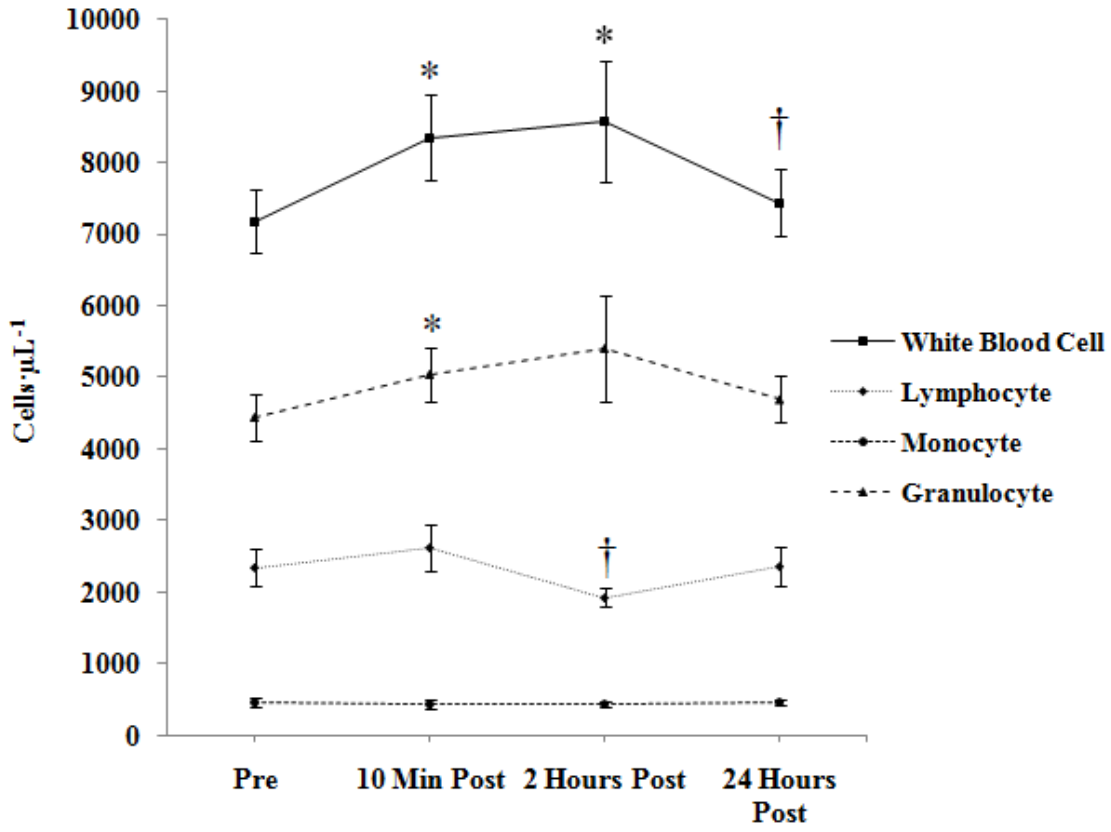
MP-Bound E-selectin is expressed as a percentage of pre-exercise levels.

Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.

There was no change in MP-bound E-selectin levels (p=0.89, Cohen's f effect size=0.14) as a result of the resistance exercise bout.

4.1.6 Haematology

Figure 4.15. Circulating Leukocyte Counts Pre- and Post- Resistance Exercise in Trained Men (n=13).



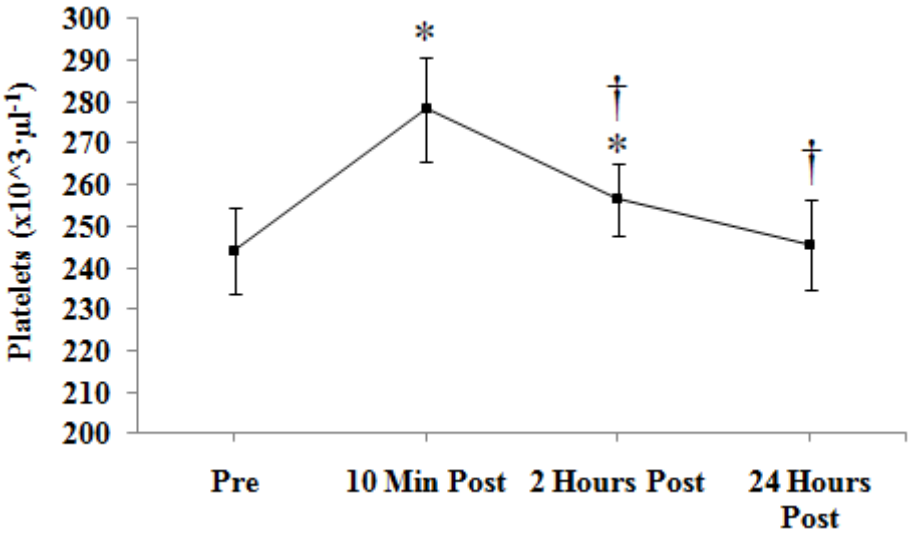
Values shown are mean ± SE

** p<0.05 significant difference from pre-exercise levels, † p<0.005 significant difference from 10 minutes post-exercise levels. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There were significant changes in total circulating leukocyte count ($p<0.05$, Cohen's f effect size=0.61), lymphocytes ($p<0.05$, Cohen's f effect size=0.49), and granulocytes

($p<0.05$, Cohen's f effect size=0.33) as a result of the resistance exercise bout. Leukocyte counts increased from pre-exercise to both 10 minutes (7176 ± 444 to 8346 ± 592 cells·μL^{-1}, $p<0.005$) and 2 hours post-exercise (7176 ± 444 to 8573 ± 854 cells·μL^{-1}, $p<0.05$), whilst returning to pre-exercise levels by 24 hours post-exercise from 10 minutes post-exercise (8346 ± 592 to 7442 ± 473 cells·μL^{-1}, $p<0.05$). Granulocyte counts increased from pre-exercise to 10 minutes post-exercise (4446 ± 327 to 5406 ± 378 cells·μL^{-1}, $p<0.05$), whereas lymphocyte count decreased from 10 minutes post-exercise to 2 hours post-exercise (2619 ± 321 to 1930 ± 123 cells·μL^{-1}, $p<0.05$). There were no significant changes in monocyte counts as a result of the resistance exercise bout ($p=0.88$, Cohen's f effect size=0.13).

Figure 4.16. Circulating Platelet Counts Pre- and Post- Resistance Exercise in Trained Men (n=13).



Values shown are mean ± SE

** p<0.05 from pre-exercise levels, † p <0.05 from 10 minutes post-exercise. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There were significant changes found in circulating platelet counts as a result of the exercise bout ($p<0.05$, Cohen's f effect size=1.20). Platelet counts significantly increased from pre-exercise to 10 minutes post-exercise (244 ± 10 to 278 ± 12 counts $x10^3 \cdot \mu L^{-1}$, $p<0.001$) and 2 hours post-exercise (244 ± 10 to 257 ± 9 counts $x10^3 \cdot \mu L^{-1}$, $p<0.005$). From 10 minutes post-exercise, platelet counts significantly declined to 2 hours post-exercise (278 ± 12 to 257 ± 9 counts $x10^3 \cdot \mu L^{-1}$, $p<0.005$) and 24 hours post-exercise (278 ± 12 to 246 ± 11 counts $x10^3 \cdot \mu L^{-1}$, $p<0.001$).

4.2 Study 2: 'The Influence of Acute Aerobic Exercise on Cell-Derived Microparticles in Trained Men'

4.2.1 Physiological Responses to Aerobic Exercise Bout

Table 4.1. Heart Rate and Blood Lactate Response to Aerobic Exercise in Trained Men (n=9).

	Pre-Exercise	Post-Exercise
Heart Rate ($b \cdot min^{-1}$)	72 ± 5	168 ± 3*
Blood Lactate ($mmol \cdot L^{-1}$)	1.6 ± 0.2	4.5 ± 0.6*

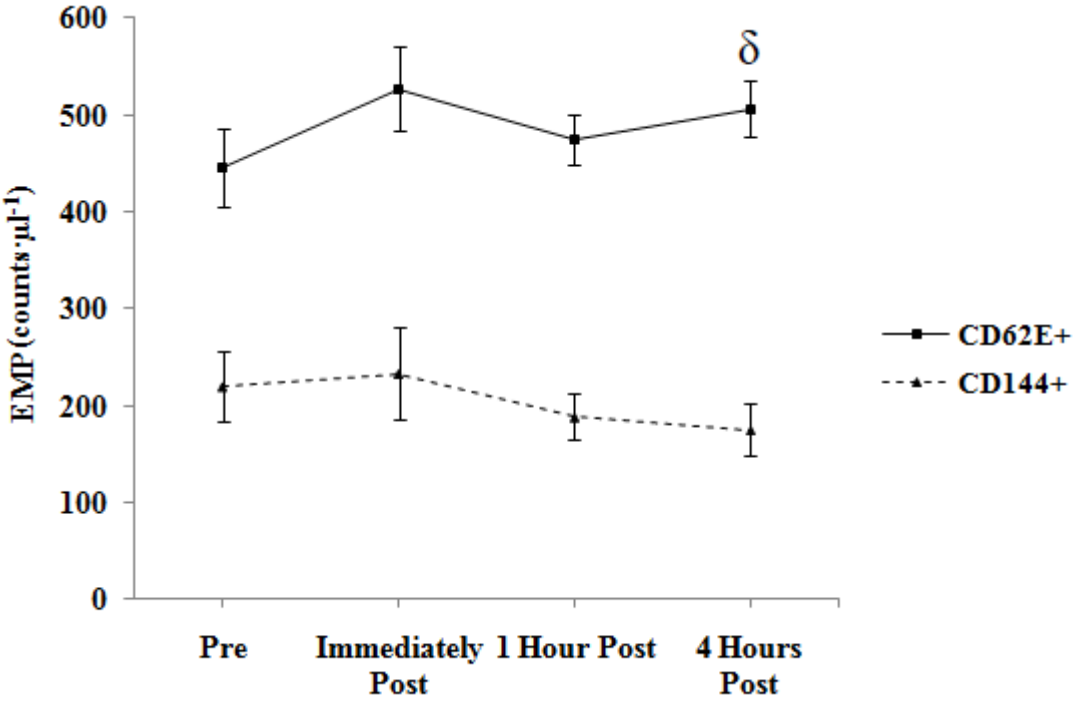
Values shown are mean ± SE

** $p<0.05$ from pre-exercise level. Data were analysed using paired samples T-test.*

The aerobic exercise bout caused an increase in subjects HR from pre- to post-exercise (72 ± 5 to 168 ± 3 $b \cdot min^{-1}$, $p<0.001$). The aerobic exercise also produced significant increase in BLa from pre- to post-exercise (1.6 ± 0.2 to 4.5 ± 0.6 $mmol \cdot L^{-1}$, $p<0.005$).

4.2.2 Cell-Derived Microparticles

Figure 4.17. Circulating Endothelial Microparticle Counts Pre- and Post- Aerobic Exercise in Trained Men (n=9).



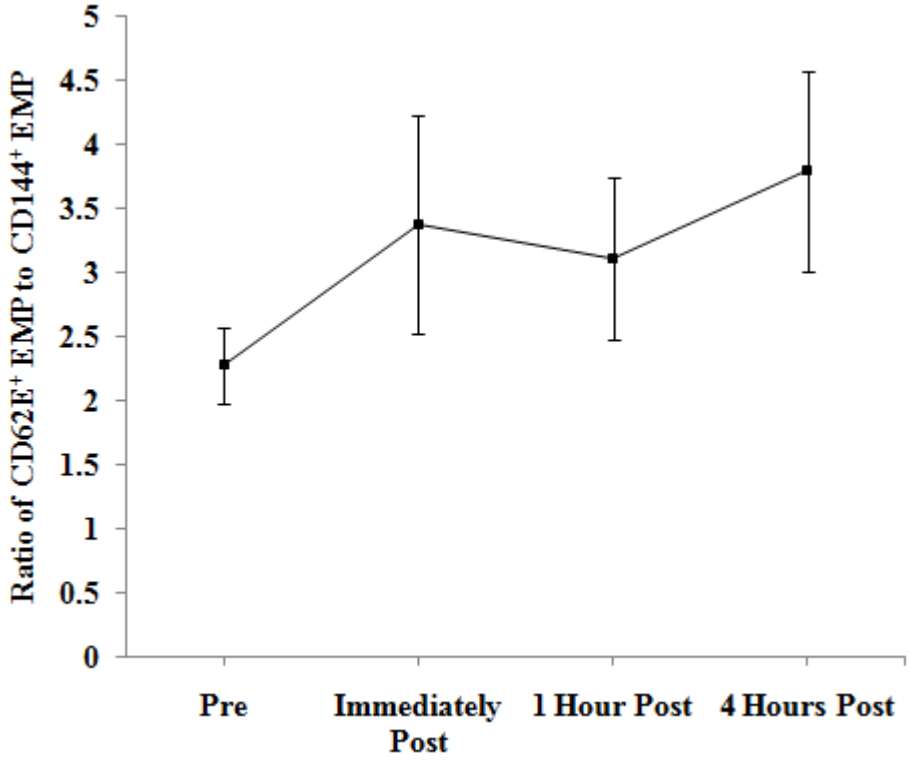
Values shown mean ± SE

δ p<0.05 significant difference from 1 hour post-exercise. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.

There was no significant difference from pre- to post-resistance exercise in $CD144^+$ EMP (p=0.30, Cohen's f effect size=0.40). However there was a significant increase in $CD62E^+$ EMP as a result of the aerobic exercise bout (p<0.05, Cohen's f effect size=0.54). An increase was found from 1 hour post-exercise to 4 hours post-exercise in

$CD62E^+$ EMPs (475 ± 26 to 506 ± 29 counts·μL^{-1} plasma, $p<0.05$). No other changes in $CD62E^+$ EMPs were evident ($p=0.078$, 0.419, 0.132 from pre-exercise to immediately post-, 1 hour post-, and 4 hours post-exercise respectively).

Figure 4.18. Ratio of CD62E$^+$ Endothelial Microparticles: CD144$^+$ Endothelial Microparticles Pre- and Post- Aerobic Exercise in Trained Men (n=9).

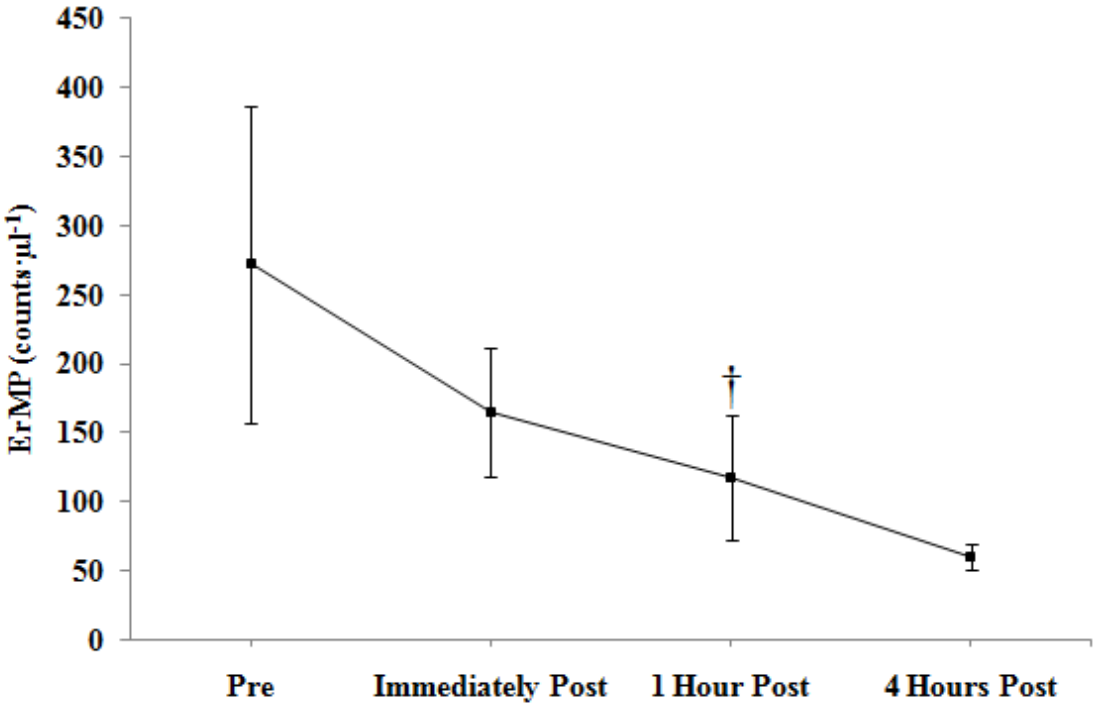


Values shown mean ± SE.

Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.

There was a change approaching significance in the ratio of CD62E$^+$ EMP to CD144$^+$ EMP as a result of the aerobic exercise bout (p=0.093, Cohen's f effect size=0.55).

Figure 4.19. Circulating Erythrocyte Microparticle Counts Pre- and Post- Aerobic Exercise in Trained Men (n=9).

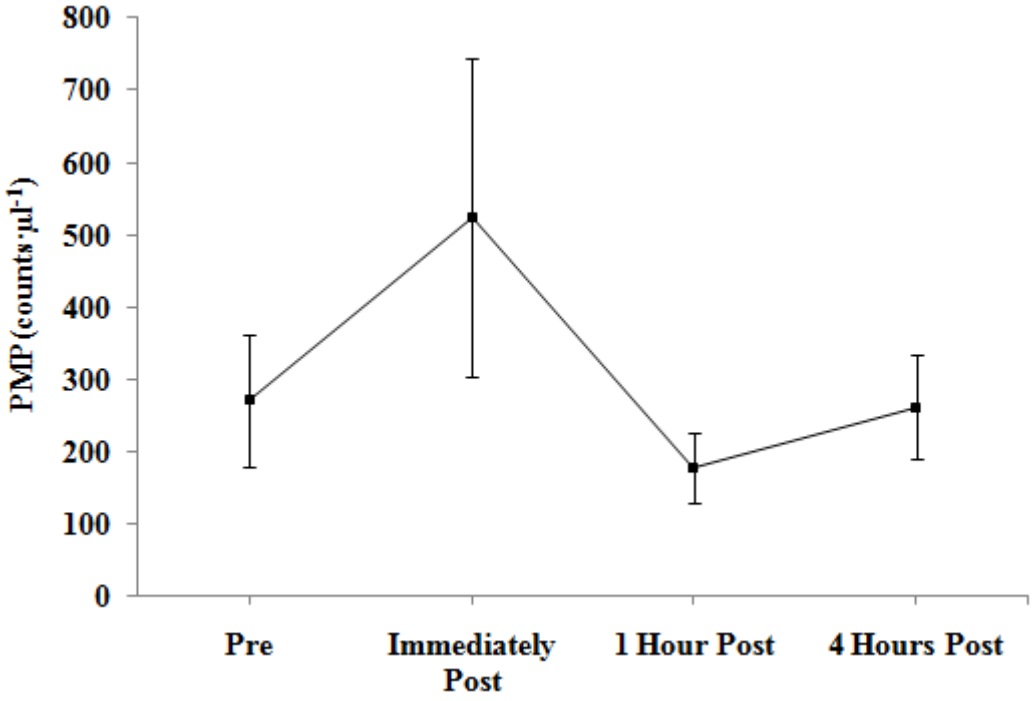


Values shown are mean ± SE.

† *p<0.05 significant difference from immediately post-exercise levels. Data were analysed using-one way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There was a significant decrease in ErMP levels as a result of the aerobic exercise bout ($p<0.05$, Cohen's f effect size=0.62). This decrease was found between immediately post-exercise to 1 hour post-exercise (165.26 ± 115.12 to 117.20 ± 45.62 counts·µL^{-1} $p<0.05$) with changes approaching significance from pre- to 1 hour post-exercise ($p = 0.072$) and from 10 minutes post- to 4 hours post-exercise ($p = 0.067$).

Figure 4.20. Circulating Platelet Microparticles Counts Pre- and Post- Aerobic Exercise in Trained Men (n=9).



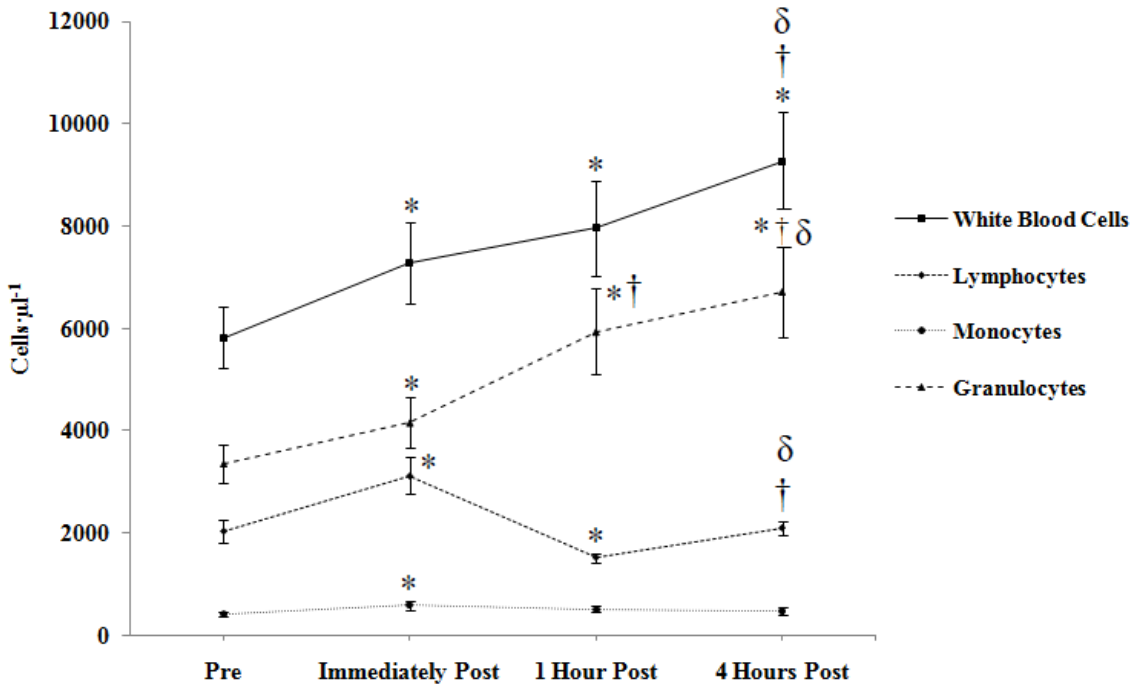
Values shown are mean ± SE.

Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.

There were no significant differences in PMP levels as a result of aerobic exercise in trained men (p=0.12, Cohen's f effect size=0.52).

4.2.3 Complete Leukocyte Counts

Figure 4.21. Circulating Leukocytes Counts Pre- and Post- Aerobic Exercise in Trained Men (n=9).



Values shown are mean ± SE

** p<0.05 significance from pre-exercise levels, † p<0.05 significance from immediately post-exercise levels, δ p<0.05 significance from 1 hour post-exercise levels. Data were analysed using one-way repeated measured analysis of variance followed by Fisher Least Significance post-hoc procedure.*

There was a significant increase in circulating total leukocytes ($p<0.05$, Cohen's f effect size=1.24), lymphotcytes ($p<0.05$, Cohen's f effect size=1.44) and granuocytes ($p<0.05$, Cohen's f effect size=1.50) as a result of the aerobic exercise bout. Leukocyte counts

increased from pre-exercise to immediately post-exercise (5817 ± 599 to 7272 ± 794 cells·μL^{-1}, $p<0.05$), 1 hour post-exercise (5817 ± 599 to 7961 ± 932 cells·μL^{-1}, $p<0.01$) and 4 hours post-exercise (5817 ± 599 to 9272 ± 958 cells·μL^{-1}, $p<0.001$). Leukocyte counts increased from immediately post-exercise to 4 hours post-exercise (7272 ± 794 to 9272 ± 958 cells·μL^{-1}, $p<0.05$), as well as from 1 hour post-exercise to 4 hours post-exercise (7961 ± 932 to 9272 ± 958 cells·μL^{-1}, $p<0.01$).

Granulocyte counts increased from pre-exercise to immediately post-exercise (3356 ± 385 to 4156 ± 489 cells·μL^{-1}, $p<0.001$), 1 hour post-exercise (3356 ± 385 to 5939 ± 845 cells·μL^{-1}, $p<0.005$) and 4 hours post-exercise (3356 ± 385 to 6722 ± 886 cells·μL^{-1}, $p<0.001$). Granulocyte counts also increased from immediately post-exercise to 1 hour post-exercise (4156 ± 489 to 5939 ± 845 cells·μL^{-1}, $p<0.05$) and 4 hours post-exercise (4156 ± 489 to 6722 ± 886 cells·μL^{-1}, $p<0.005$), and from 1 hour post-exercise to 4 hour post-exercise (5939 ± 845 to 6722 ± 886 cells·μL^{-1}, $p<0.05$).

Lymphocyte counts increased from pre-exercise to immediately post-exercise (2033 ± 238 to 3128 ± 357 cells·μL^{-1}, $p<0.01$), and decreased to below baseline 1 hour post-exercise (2033 ± 238 to 1511 ± 92 cells·μL^{-1}, $p<0.05$). Lymphocyte counts decreased from immediately post-exercise to 1 hour post-exercise (3128 ± 357 to 1511 ± 92 cells·μL^{-1}, $p<0.001$). Lymphocyte counts at 4 hours post-exercise was significantly lower than immediately post-exercise (3128 ± 357 to 2100 ± 127 cells·μL^{-1}, $p<0.01$), but raised from 1 hour post-exercise (1511 ± 92 to 2100 ± 127 cells·μL^{-1}, $p<0.001$).

Monocyte counts were not significant changed as a result of the aerobic exercise bout ($p=0.10$, Cohen's f effect size=0.54).

Chapter 5

Discussion and Conclusion

5.0 Discussion and Conclusion

The main objectives of the study were to investigate the effects of an acute bout of resistance exercise on circulating endothelial progenitor cells (EPC) as well as cell-derived microparticles (MP) of endothelial (EMP), platelet (PMP) and erythrocyte (ErMP) origin. Measurements were taken pre-exercise and then at 10 minutes, 2 hours and 24 hours post-exercise. The exercise bout employed low intensity resistance with high repetitions in order to stimulate tissue hypoxia and metabolic acidosis. A number of growth factors were also measured to explain potential changes in EPCs. Secondary to this was to investigate the effects of an acute bout of aerobic exercise on cell-derived MPs (EMP, PMP and ErMP).

5.1 Study 1: 'The Influence of Acute High Volume Low Intensity Resistance Exercise on Circulating Endothelial Progenitor Cells and Cell-Derived Microparticles in Trained Men'

5.1.1 Endothelial Progenitor Cells

EPCs increased at various timepoints post exercise whether expressed as raw cell counts (cells per mL whole blood; figure 4.3), as percentage of total $CD45^+$ events (figure 4.6), or as a percentage of total progenitor cells (figure 4.6). This increase was not present immediately post-exercise but evident at 2 and 24 hours post-exercise. The greatest increase appears to be at 24 hours post-exercise; however this is only significant when EPCs are expressed as a percentage of total events (figure 4.5) or total progenitor cells

(figure 4.6). The increase in raw cell counts was ~62% at 2 hours post-exercise, and ~91% at 24 hours post-exercise. This increase appears to have occurred in the absence of a change in total circulating progenitors (figure 4.4), representing an increase in differentiation of progenitors towards an endothelial lineage rather than an increase in total progenitors. However caution must be observed with this assertion due given that the change in total progenitors was of moderate effect size, despite the lack of statistical significance.

Although this is the first study that has examined the influence of resistance exercise on circulating EPCs, the results are largely similar to the changes observed in EPCs following a bout of aerobic exercise (Adams *et al*., 2004; Rehman *et al*., 2004; Laufs *et al*., 2005; Van Craenenbroeck *et al*., 2008; Goussetis *et al*., 2009; Möbius-Winkler *et al*., 2009; Thorell *et al*., 2009; Bonsignore *et al*., 2010; Sandri *et al*., 2011; Van Craenenbroeck *et al*., 2011). The majority of previous studies show increases in EPCs immediately post-exercise (Rehman *et al*., 200; Laufs *et al*., 2005; Van Craenenbroeck *et al*., 2008; Goussetis *et al*., 2009; Mobius-Winkler *et al*., 2009; Bonsignore *et al*., 2010; Van Craenenbroek *et al*., 2011). Of these studies, those that examined EPCs over a 24-72 hour period, the majority observed a return to baseline within 24-48 hours post-exercise (Möbius-Winkler *et al*., 2009; Bonsignore *et al*., 2010). The range of increases are within the range of 2 to 10 fold increase. It is important to note that a number of studies did not measure EPCs after 2 hours post-exercise (Rehman *et al*., 2004; Laufs *et al*., 2005; Shaffer *et al*., 2006; Adams *et al*., 2008; Van Craenenbroeck *et al*., 2010) making comparisons with the current study difficult.

5.1.1.2 Mechanisms for Resistance Exercise Effect on Endothelial Progenitor Cells

The mechanisms by which circulating EPCs levels increase following a bout of resistance exercise are open to debate. The resistance bout was anaerobic, as identified by the high blood lactate (BLa) levels (figure 4.2). Therefore there may be increases in hypoxia-inducible factor-1 (HIF-1) which is a transcription factor that is associated with hypoxic and anaerobic exercise (Richardson *et al*., 1995; Ameln *et al*., 2005). Hypoxia results in increased levels of VEGF (Sheiki *et al*., 1992). There was a clear increase in VEGF at 10 minutes post-exercise (figure 4.7) which may mobilise EPCs from the bone marrow (BM) as a result of chemoattraction since the EPCs we measured expressed a receptor for VEGF (VEGFR2). Indeed VEGF has been found to increase along with EPC levels as a result of exercise (Adams *et al*., 2004; Möbius-Winkler *et al*., 2009; Sandri *et al*., 2011). Asahara *et al* (1999a) suggests that they may also enhance the proliferative effect of these EPCs within the BM, thus causing subsequent leaking into the circulation, or by enhancing vascular permeability and modulating the adhesion molecule expression on the BM endothelium. However, VEGF alone may not be able to explain increases in EPCs unless effects are delayed.

To our knowledge this is the first study to show an accompanied increase in granulocyte-colony stimulating factor (G-CSF) with EPCs as a result of an exercise bout (figure 4.8). Morici *et al* (2005) found no such increase in G-CSF and so concluded that the increase in haematopoietic stem cells were not a result of G-CSF-induced mobilisation as a result of aerobic exercise. It may be that there may be a modality-specific response with G-CSF. It is unclear which tissues are responsible for the

increased G-CSF observed at 2 hours post-exercise. G-CSF has been found to be released by activated endothelial cells (EC) (Zsebo *et al*., 1988), and human fibroblasts (Fibbe *et al*., 1988). G-CSF has been observed to induce BM–derived stem cell mobilisation by causing a release of elastase from neutrophils, which goes on to degrade stromal-derived factor-1 (SDF-1) within the BM, allowing for stem cells to move in to the circulation (Petit *et al*., 2002). G-CSF also acts in a similar fashion to mobilise granulocytes from the BM (Yamada *et al*., 2002), hence its name. We observed increases in granulocytes post-exercise following resistance training.

It may also be the case that some of these progenitor cells released the soluble factors VEGF and G-CSF as angiogenic regulators, as they have shown to be able to do so previously (Hur *et al*., 2004). It is possible that other factors such as IL-6, insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF) which have not been measured in this study contribute to resistance exercise-mediated circulating EPC increase.

It is also possible that the increase in EPCs was independent of hypoxia. IGF-1 is a growth factor whose expression has been shown to be increased after a bout of resistance exercise (Haddad & Adams, 2002). IGF-1 is important for the hypertrophic adaptation of skeletal muscle as a result of resistance exercise training (Philippou *et al*., 2007). Interestingly, IGF-1 has been found to be a modulator of capillary tube formation by BM-derived cells (Nakamura *et al*., 2010), as well as modulators of SDF-1-induced angiogenesis (Sengupta *et al*., 2010) and increases in serum IGF-1 resulted in increases in circulating EPCs in healthy adults (Devin *et al*., 2008). Therefore it is possible for IGF-1 to be released after an acute bout of resistance exercise, stimulate increased SDF-

1 expression and production, causing the release of BM-derived EPCs from the BM. IGF-1 was not measured in this study however.

It is unlikely that inflammation significantly contributed to an increase in circulating EPCs as a result of this bout of resistance exercise as although there was an increase in circulating leukocytes immediately post-exercise (figure 4.15), this increase was not present 24 hours post-exercise, suggesting only a minor inflammatory response. Goussetis *et al* (2009) found a long duration exercise bout induced increases in EPC that was accompanied by an increase in the inflammatory marker C-reactive protein (CRP). These have not been measured in this study therefore we cannot exclude the possibility of inflammation having a significant effect on EPC mobilisation.

It is possible that the higher EPC values observed at 2 hours post-exercise was not the result of increased EPC production and differentiation in the BM but rather then release of EPCs from marginal pools where they are tethered to ECs. It is known that leukocytes are released post-exercise as a result of the increased levels of adrenaline and noradrenaline due to their expression of adrenergic receptors (Lanmann, 1992; Benschop *et al*., 1996; Schedlowski *et al*., 1996) and this may be the case for EPCs. However we did not measure either circulating levels of these catecholamines, nor did we measure adrenergic receptor expression on EPC surface membranes. Increased shear stress may also have played a role here resulting in mechanical disruption. Increases in subjects' heart rate in this study (figure 4.1) indicate increased cardiac output, thus increases in blood flow. However a number of factors argue against this hypothesis. These are that the EPC increase is highest at 24 hours post-exercise when and if we expect mechanical disruption to be a factor in EPC release then we would expect peak EPC to be immediately post-exercise.

Further studies are needed in order to further elucidate the mechanisms behind the increase in circulating EPCs as a result of resistance exercise.

The increases in circulating EPCs following an acute bout of resistance exercise has implications for individuals who exercise for health purposes, but also for athletes who are attempting to induce angiogenesis to improve blood flow capacity to the skeletal muscle. This study adds to a volume of evidence suggesting resistance exercise has health benefits and so justifies the emphasis on resistance exercise in current guidelines. For those who are interested in adaptations for athletes taking part in endurance events, high volume low intensity resistance exercise is warranted.

5.1.2 Microparticles

$CD62E^+$ EMPs increased by 22% as a result of resistance exercise (figure 4.9). This increase was only significant at the 24 hours post-exercise timepoint. Soluble E-selectin also increased as a result of resistance exercise, an increase which approached significance (figure 4.13). These are both markers of endothelial activation; therefore we can assume that resistance exercise caused an activation of the endothelium. There is no evidence to suggest the increases in $CD62E^+$ EMP and soluble E-selectin result from different triggers. When MP-bound E-selectin was assessed in a novel immunoassay under development in our lab, there was no similar increase (figure 4.14). There were no changes in $CD144^+$ EMP (figure 4.9).

Despite the absence of a change in MP-bound CD62E in our developmental assay, the changes in $CD62E^+$ EMP, the ratio of $CD62E^+$ EMP to $CD144^+$ EMP and the changes approaching significance in soluble E-selectin all suggest endothelial activation following resistance exercise. The absence of changes in $CD144^+$ EMP suggest that EC

apoptosis was unchanged. E-selectin/CD62E is not present on EC membranes in high concentrations in the rested unactivated state but is translocated there during EC activation. Thus increases in $CD62E^+$ EMP or indeed soluble E-selectin probably represents increasing E-selectin expression on the EC membrane following resistance exercise. Only one previous study has observed an increase in EMP levels as a result of exercise (Sossdorf *et al*., 2011) however the majority of studies have observed no such increases in EMP as a result of acute aerobic exercise (Möbius-Winkler *et al*., 2009; Chen *et al*., 2010; Sossdorf *et al*., 2010; Chaar *et al*., 2011). This is most likely due to several reasons; the definition of EMPs (defined as either $CD62E^+$, $CD144^+$ or $CD106^+$), the plasma processing procedure (e.g. the spin protocol and time of incubation with antibodies), the subject population recruited (healthy and diseased individuals as well as trained and untrained), the timepoints of samples taken, and the exercise bout (duration and intensity).

One possible mechanism for the increase in EMP as a result of exercise is inflammation. As mentioned previously, our leukocyte data indicated no significant inflammatory response 24 hours post-exercise (figure 4.15), the same time when $CD62E^+$ EMPs were at their highest. Since we have not measured any cytokines shown to be markers of inflammation we cannot exclude the possibility of inflammatory markers having a significant effect on EMP levels. Another possible mechanism is an increase in shear stress. Shear stress may cause EMP release through disruption of the EC cytoskeleton, which is a pivotal role in MP formation (Yano *et al*., 1994). Exercise-induced increases in shear stress may exert a protective role on the endothelium. By initial exposure to shear stress, ECs exhibit increased levels of activation and apoptosis, and possible release of EMPs, but due to regular exposure to shear stress, these ECs can be replaced

by apoptotic-resistant ECs (Sakao *et al*., 2005). With resistance exercise there is a post-contraction transient increase in shear stress (Rakobowchuk *et al*., 2005), which may be of greater magnitude to that of aerobic exercise and thus contributed to the increase in $CD62E^+$ EMP 24 hours post-exercise. However, since we did not measure this directly we cannot conclude that the response was due to shear stress alone.

Interestingly we found no change in $CD41^+$ annexin V^+ PMP as a result of resistance exercise (figure 4.12). PMPs have previously been consistently found to be increased as a result of exercise (Sossdorf *et al*., 2010; Chaar *et al*., 2011; Sossdorf *et al*., 2011), thought to be due to shear stress (Chen *et al*., 2010). Furthermore, a decline approaching significance was found with circulating $CD235a^+$ ErMP (figure 4.11). This is the first study to show this effect. The increase in circulating leukocytes post-exercise (figure 4.15) may have resulted in increased phagocytosis of these MP subsets.

5.1.3 Interaction of EPC and EMP Changes

The parallel increases in EPC (figure 4.3) and $CD62E^+$ EMP (figure 4.9) potentially represent a co-ordinated response to resistance exercise. Interestingly, E-selectin has been found to aide in the binding of EPCs to the endothelium (Oh *et al*., 2007), and may play role in exercise-induced binding as exercise has previously been shown to increase soluble E-selectin levels (Boos *et al*., 2008). EPCs facilitate angiogenesis and repair, and increased E-selectin on EC membranes facilitate the binding of these regenerative cells (Oh *et al*., 2007). In fact soluble E-selectin has been found to promote EC tube formation *in vitro* (Yasuda *et al*., 2002) and promotes EC chemotaxis *in vitro* (Koch *et al*., 1995). Therefore, EPCs, $CD62E^+$ EMPs, and soluble E-selectin may play a collective role in exercise-induced angiogenesis.

5.2 Study 2: 'The Influence of Acute Aerobic Exercise on Cell-Derived Microparticles in Trained Men'

5.2.1 Cell-Derived Microparticles

The main finding from this study was that there was a significant 6.5% increase from 1 hour post-exercise to 4 hours post-exercise in $CD62E^+$ EMP (figure 4.17), with a change that approached significance from pre- to immediately post-exercise (~18%, p=0.078). There were no accompanying changes in circulating levels of $CD144^+$ EMP (figure 4.17), suggesting that aerobic exercise causes activation of the endothelial layer rather than apoptosis, similar effect seen with the resistance exercise bout in study 1. Further evidence of EC activation is seen with a change approaching significance from pre-exercise to 4 hours post-exercise in the ratio of $CD62E^+$ EMP: $CD144^+$ EMP (figure 4.18). The mechanisms behind this response may be due to several reasons. As mentioned previously, an increase in cardiac output as a result of aerobic exercise will result in an increase in shear stress. Shear stress may have caused mechanical activation of the EC wall, thus causing a possible disruption of the EC cytoskeleton, which is a crucial process in the formation of MPs (Yano *et al*., 1994). As mentioned previously, exercise-induced increases in shear stress may exert a protective role on the endothelium. Initially, however, ECs exhibit increased levels of apoptosis, and probably release of EMPs into the circulation, but as this stimulus becomes more common ECs can be replaced by apoptotic-resistant ECs (Sakao *et al*., 2005). However, there may be other underlying factors that are known to cause significant EC activation that were not measured in this study and could have been raised in the exercise bout, for example oxidative stress and heat stress (Marsh & Coombes, 2005).

As with the resistance exercise study there was no change observed with PMPs (figure 4.20) as seen in other studies (Sossdorf *et al*., 2010; Chaar *et al*., 2011; Sossdorf *et al*., 2011). However there was a clear trend towards an increase immediately post- exercise. The possible issue of a lack of statistical power as a result of low subject numbers may be masking this increase. ErMPs were found to be decreased as a result of the running bout (figure 4.19). This may be due to an increase in circulating leukocytes that may phagocytose the 'debris' thought to be caused by shear stress.

This is only the second study to date that has shown an increase in EMP as a result of aerobic exercise, the first being published recently (Sossdorf *et al*., 2011). However the majority of studies have found no increases in EMPs (Möbius-Winkler *et al*., 2009; Chen *et al*., 2010; Sossdorf *et al*., 2010; Chaar *et al*., 2011). The disparity between this result and other seen in other studies are most probably due to a wide range of EMP definitions being used in these studies, the differences in the exercise bout (intensity and duration), as well as the participants being recruited for the study, whether they be trained or untrained, or have a history of cardiovascular risk factors. For example, Sossdorf *et al* (2010, 2011) subjected participants to a 90 minute cycling bout at 80% of individual anaerobic threshold, with CD62E being a marker of EMPs, and PMPs measured as expressing CD42a, whereas Chen *et al* (2010) observed no change in PMP (defined as $CD61^+$) after a graded exercise test, and Chaar *et al* (2011) found that with a completely different exercise protocol (intermittent submaximal and maximal exercise) EMP ($CD106^+$) and ErMP ($CD235a^+$) did not change, whereas $CD41^+$ PMP were found to be increased. This highlights the need for a standard definition for MP subsets in order to fully understand the effects of exercise on the endothelium, platelets and erythrocytes.

5.3 Directions for Future Studies

This study has shown an increase in EPCs as a result of an acute bout of resistance exercise. Considering the possible vascular responses as a result of an acute bout of exercise, exercise training studies should be performed to observe chronic adaptations that may have both health and sporting performance benefits. These studies could fully elucidate the mechanisms behind both resistance and aerobic exercise-induced angiogenesis, crucial for improved blood supply to the working muscles for sporting performance, especially endurance performance, as well as understanding how regular exercise may help in restoring blood flow to ischemic muscle in those with ischemic cardiovascular disease.

This study has shown an increase in EPC number, further studies should include functional measures of EPCs and vascularisation as a result of exercise bouts. Functional assays are now becoming the focus of EPC biology, and therefore cell enumeration by flow cytometry, although valid, does not reflect functional changes as a result of a stimulus or a stressor. For example, functional measures such as migration, tube formation and induced angiogenic growth factor secretion assays should be performed.

The effects of training on acute exercise-induced responses should also be considered, when questions regarding whether EPCs will be mobilised to the same extent or whether the EC layer will have developed a protective mechanism in order to prevent damage or activation as a result of the same exercise could be answered. Otherwise comparisons in the response of EPCs and EMPs as a result of acute resistance and aerobic exercise can be made in trained and untrained subjects. Since this study

investigated resistance exercise, the comparison between modalities of exercise should be made, whereby subjects participate in both resistance and aerobic exercise and the responses compared in EPC number and/or function and MP number.

5.4 Limitations

This study is not without its limitations. Only selected markers were measured to explain changes in EPCs, whereas there are a host of potential markers that could explain these changes. Further analyses should include specific pro-inflammatory cytokines (CRP), markers of muscle damage (creatine kinase) and other growth factors that have been suggested to increase circulating EPC levels including bFGF and IGF-1. In addition, in the aerobic exercise study the sample size may not have been sufficient enough for some results to reach statistical significance, therefore more subject numbers above the pre-determined power sample size could have potentially unmasked any changes in MPs as a result of exercise. There were no significant changes in various markers despite a moderate to large effect size. Specifically, the effect size for the change in circulating progenitor cells, ErMPs, and soluble E-selectin in the resistance study and $CD144^+$ EMP, the ratio of $CD62E^+$ EMP:$CD144^+$EMP and PMP in the aerobic exercise study all failed to reach statistical significance, despite an effect size > 0.30. This suggests that there is a moderate or large change, which would have reached significance if there were additional subjects recruited to the study. However, it is important to note that significant changes in our main population markers (EPCs and EMPs) were observed with the subject population that were recruited.

5.5 Conclusion

The principal finding of this study was an increase in circulating EPCs following a bout of low intensity resistance exercise. This occurred in the absence of any change in total circulating progenitor cells. The increase in EPCs was accompanied by an increase in $CD62E^{+}$ EMP, and an increase in soluble E-selectin that approached significance, both markers of endothelial activation. VEGF and G-CSF were also elevated at various timepoints post-exercise. To our knowledge this is the first study to demonstrate this effect. This finding is in line with previous studies which demonstrate bouts of aerobic exercise can cause an increase in circulating EPC levels (Adams *et al*., 2004; Rehman *et al*., 2004; Laufs *et al*., 2005; Van Craenenbroeck *et al*., 2008; Goussetis *et al*., 2009; Möbius-Winkler *et al*., 2009; Thorell *et al*., 2009; Bonsignore *et al*., 2010; Sandri *et al*., 2011; Van Craenenbroeck *et al*., 2011). There has only been one study to date that demonstrates an increase an increase in EMPs as a result of exercise (Sossdorf *et al*., 2011), yet this is the first to report an increase as a result of a bout of resistance exercise.

In summary an acute bout of resistance exercise increases circulating levels of both EPCs and EMPs. These changes may represent a co-ordinated response so that the mobilised EPCs have additional opportunities to bind to the endothelium. The increases in EPC are associated with increases VEGF and G-CSF. A short bout of resistance exercise may be a time-efficient mode of exercise by which to improve cardiovascular health and possibly enhance cardiovascular adaptation for sports performance.

Chapter 6

References

6.0 References

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Appendices

Appendix A

Waterford Institute *of* Technology
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Project Information Sheet

Background

The endothelium makes up the inner layer of all blood vessels and is now know to perform a range of crucial functions related to cardiovascular health. There is considerable interest amongst scientists in endothelial microparticles and endothelial progenitor cells as markers of endothelial stress and endothelial regeneration. Endothelial microparticles are fragments of endothelial cells shed into the bloodstream in response to cell stress and injury. Endothelial progenitor cells are a type of stem cell involved in the regeneration of areas of endothelial damage and the growth of new blood vessels. These novel markers provide exercise scientists with a means of studying the effects of exercise on the endothelium (normally inaccessible) via a simple blood sample.

Purpose of the study

The purpose of the study is to compare the effects of an acute bout of exercise on biomarkers of endothelial damage and endothelial regeneration, in trained and untrained men.

Procedures

As a subject in this project, you will be asked to refrain from any strenuous exercise 3 days prior to any exercise testing. You will also be asked to refrain from ingesting any caffeine and alcohol the day prior to exercise testing.

You will be asked to take part in a maximal exercise test ($\dot{V}O_2$max), as well as at least one of the following:

Aerobic exercise

Anaerobic exercise

Resistance exercise

Blood samples will be taken immediately pre- and post-, 1 hour and 4 hours post-exercise.

All information will be kept strictly confidential.

Appendix B

Waterford Institute *of* Technology
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Physiology Screening Questionnaire

Please read the following carefully and answer all the questions truthfully.

Information will be treated with the strictest confidence.

This is a strictly private confidential document.

Name: Gender:

Date of Birth: Age:

Have you ever had a heart problem such as a heart attack, hypertrophic cardiomyopathy, congenital abnormality, heart valve defect, heart failure or heart rhythm disturbance?	Yes/No
Have you ever received treatment for a heart problem such as heart surgery, the fitting of a pacemaker/defibrillator, coronary angioplasty or heart transplantation?	Yes/No
Are you currently taking medication for your heart?	Yes/No
Do you currently or have you ever suffered from any of the following:	
Arthritis, osteoporosis or any other bone or joint problem?	Yes/No
Asthma, bronchitis or any other respiratory problem?	Yes/No
Coagulation disorders?	Yes/No
Diabetes (Type I or Type II)?	Yes/No
Epilepsy?	Yes/No

Hypertension (High Blood Pressure)?	Yes/No
Liver or gastrointestinal problems?	Yes/No
Kidney problems?	Yes/No
Infectious disease such as HIV, hepatitis or glandular fever?	Yes/No

Do you experience any of the following:

Chest discomfort with exertion?	Yes/No
Unreasonable breathlessness?	Yes/No
Dizziness, fainting, blackouts?	Yes/No
Palpitations or skipped heart beats?	Yes/No
Unusual levels of fatigue?	Yes/No

Please indicate if any of the following are true:

You have a close blood male relative (father or brother) who has had a heart attack before the age of 55 or a close female relative (mother or sister) who has had a heart attack before the age of 65?	Yes/No
You have elevated levels of cholesterol or are on lipid lowering medication?	Yes/No
You are a cigarette smoker or have quit within the last 6 months?	Yes/No
You have elevated levels of blood glucose?	Yes/No
You are completely inactive (do not take part in 20 minutes of moderate physical activity such as walking, 3 times per week)	Yes/No
Are you currently taking any medications?	Yes/No

If Yes please give details:

Have you any other conditions that may be relevant to an individual undertaking strenuous exercise? Yes/No

If Yes please give details:

_____

Physical Measurements

Blood Pressure _____ mmHg

BMI _____ kg/m^2

Declaration:

I have under stood all of the questions put to me and that my answers are correct to the best of my knowledge. I understand that this information will be treated with the strictest confidence.

Signed: _____ Date: _____

(Participant)

Signed: _____ Date: _____

(Tester)

Appendix C

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RESEARCH - INFORMED CONSENT FORM

I. Project Title:

Influence of acute exercise on circulating microparticles and endothelial progenitor cells in trained and untrained men

II. Introduction to this study:

The endothelium makes up the inner layer of all blood vessels and is now know to perform a range of crucial functions related to cardiovascular health. There is considerable interest amongst scientists in endothelial microparticles and endothelial progenitor cells as markers of endothelial stress and endothelial regeneration. Endothelial microparticles are fragments of endothelial cells shed into the bloodstream in response to cell stress and injury. Endothelial progenitor cells are a type of stem cell involved in the regeneration of areas of endothelial damage and the growth of new blood vessels. These novel markers provide exercise scientists with a means of studying the effects of exercise on the endothelium (normally inaccessible) via a simple blood sample.

III. I am being asked to participate in this research study. The study has the following purposes:

1. To determine the influence of different forms of exercise on circulating microparticles and endothelial progenitor cells in young men

2. To compare the exercise response in trained and untrained men

IV. This research study will take place at Waterford Institute of Technology

V. This is what will happen during the research study:

You will undertake initial measurements in the exercise laboratory that include assessment of maximum aerobic fitness capacity, maximum strength levels and percentage body fat.

You will undertake three different forms of exercise in the laboratory on three different days, an aerobic session, a sprint session and a resistance training session. Each session will last 30 - 40 minutes. A blood sample will be taken from a forearm vein immediately prior to exercise, immediately after exercise, and at 2 hours, and 24 hours post-exercise. These samples will later be analysed for markers related to cardiovascular health. Approximately 15 mL (three teaspoonfuls) of blood will be taken during each sample.

You will be asked to refrain from exercise training and not to consume alcohol on the day prior to each exercise session. You will also be asked to follow a similar diet for one day prior to each test.

VI. There are certain risks and discomforts associated with participation in the study

1. Strenuous exercise carries with it a very small risk of heart attack. This risk only exists for a small number of individuals with pre-existing heart problems. Every effort will be made through pre-exercise screening to identify individuals with heart and other conditions that could be made worse with exercise. A defibrillator is always on site in the laboratory.

2. Fatigue will be experienced during the exercise sessions. In addition, muscle soreness may be experienced for a day or two after exercise, particularly after the resistance training session.

3. A small amount of localised bruising can occur after a blood sample is taken. Every effort will be made to avoid this by the individual taking the blood sample.

VI. My confidentiality will be guarded:

Waterford Institute of Technology will protect all the information about me and my part in this study. My identity or personal information, will not be revealed, published or used in future studies. The study findings will form the basis for preparation of a postgraduate thesis, academic publications, conference papers and other scientific publications.

VII. If I have questions about the research project, I am free to call Dr. Michael Harrison at telephone no. 051-302161:

VIII. Taking part in this study is my decision.

If I do agree to take part in the study, I may withdraw at any point. There will be no penalty if I withdraw before I have completed all stages of the study. In the case of WIT students, academic grades and progress will not be affected in any way. However, once I have completed the study I will not be allowed to have my personal information and results removed from the database.

IX. Signature:

I have read and understood the information in this form. My questions and concerns have been answered by the researchers, and I have a copy of this consent form. Therefore, I consent to take part in this research project entitled: *"Influence of acute exercise on circulating microparticles and endothelial progenitor cells in trained and untrained men"*

Signed: _____

Date: _____

Witness:_____

Signature

Appendix D

Endothelial Progenitor Cell Gating Strategy

The gating strategy by which we enumerate EPCs is also a matter of debate. Firstly, to ensure that the sample was properly lysed, a graph of FS vs. SS was observed (figure A.1). For this study, $CD45^+$ cells were gated (gate A; figure A.2), and this gate applied to SS vs CD34-PC7 graph, and subsequently a population of $CD34^+$ cells with low SS properties were gated (gate B; figure A.3) as recommended (Sutherland *et al*., 1996).

Figure A.1. Forward Scatter versus Side Scatter Colour Dot Plot for Lysed Whole Blood.

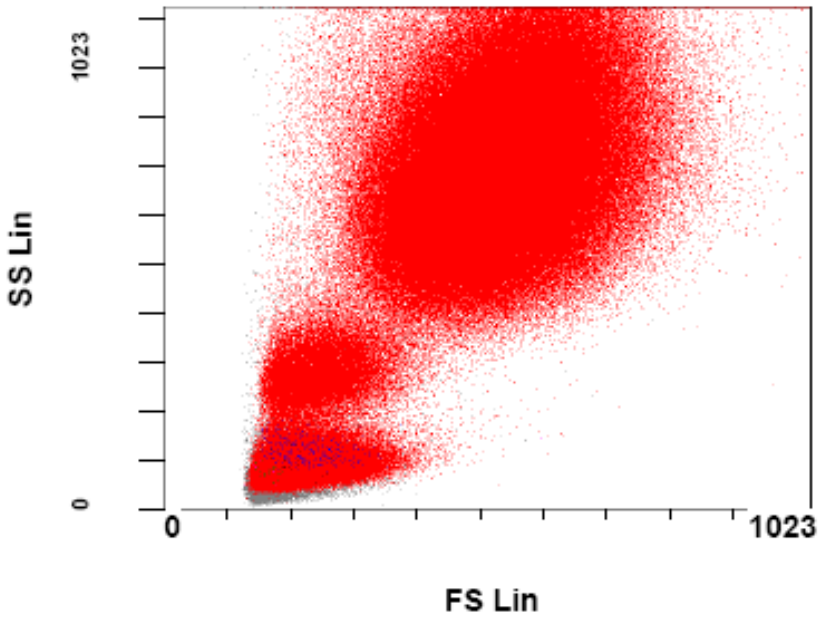


Figure A.2. Colour Dot Plot of Side Scatter vs. CD45-FITC using Lysed Whole Blood.

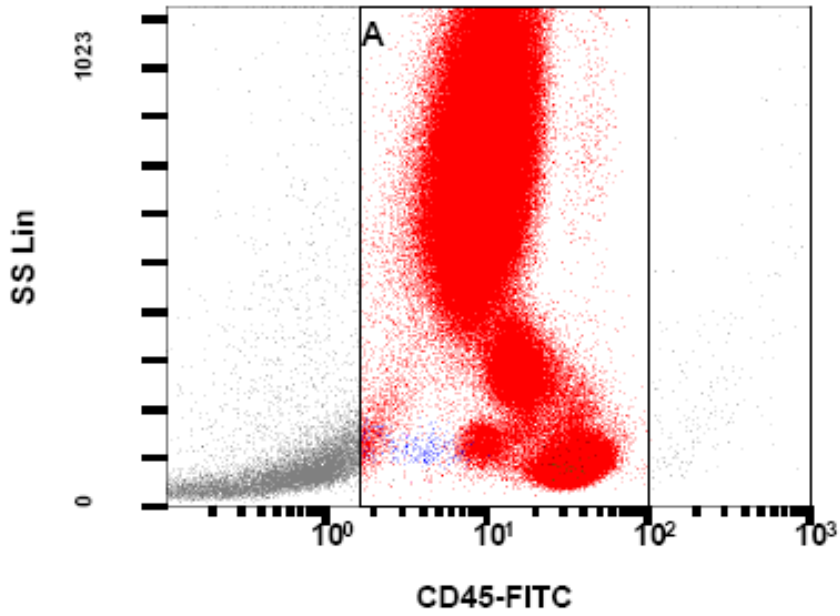
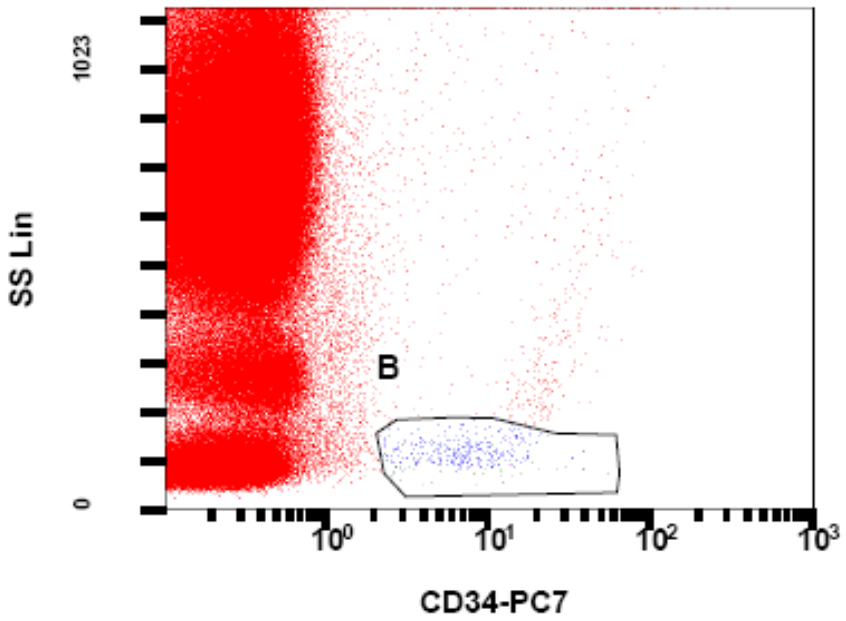
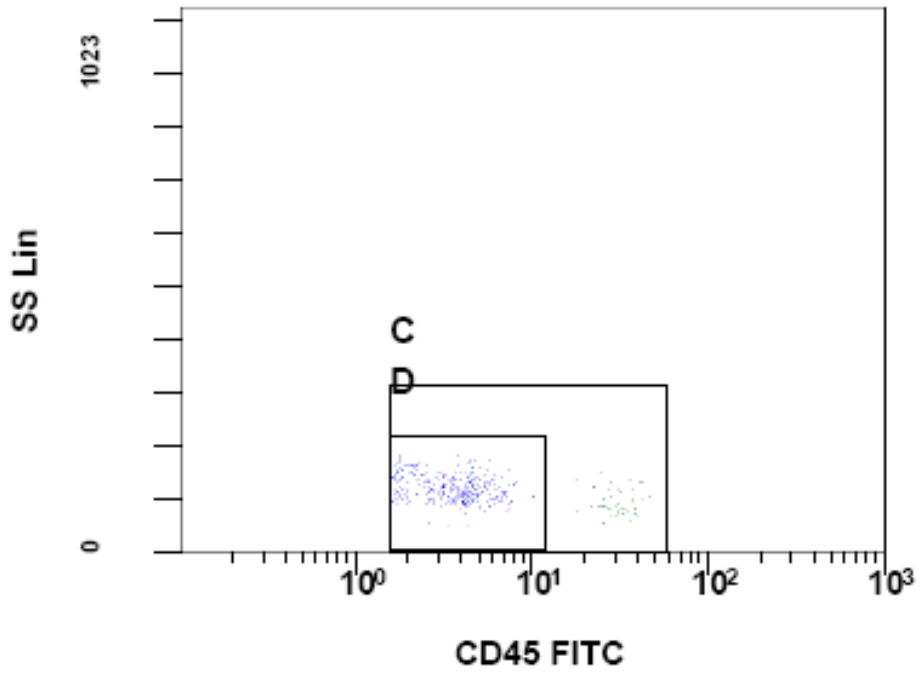


Figure A.3. Colour Dot Plot of Side Scatter vs. CD34-PC7 in Lysed Whole Blood Gated on Gate A.



Gate B was applied to another graph of SS vs CD45 and two populations of cells were identified. One population had low $CD45^{+}$ signal strength. These cells will be termed $CD45^{dim}$ cells. A second population were highly positive for CD45. To analyse $CD34^{+}VEGFR2^{+}$ and $CD34^{+}VEGFR2^{+}CD45^{dim}$ cells (both are reported definitions of EPCs), two gates were used: C and D respectively (figure A.4).

Figure A.4. A Second Colour Dot Plot of Side Scatter vs. CD45-FITC in Lysed Whole Blood Gated on Gate B.



Gate C or D (depending on definition of EPC) was applied to the first graph of FS and SS. Reportedly, EPCs are found within the lymphocyte gate, so in order to exclude any events outwith the lymphocyte region, gate E was drawn (figure A.5) and applied to a colour dot plot of CD34-PC7 vs. VEGFR2-PE (figure A.6).

Figure A.5. Colour Dot Plot of Side Scatter vs. Forward Scatter in Lysed Whole Blood Gated on C or D.

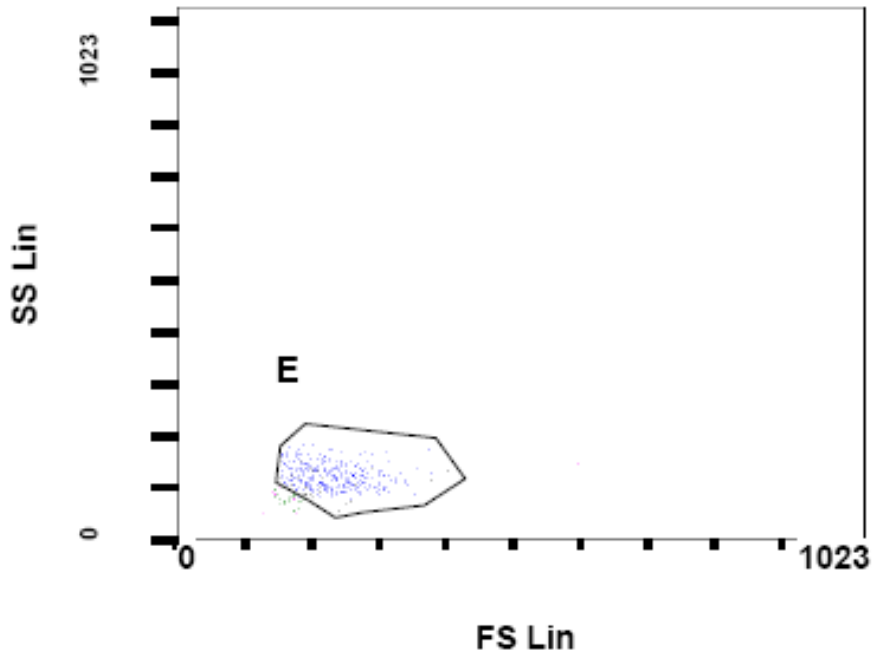
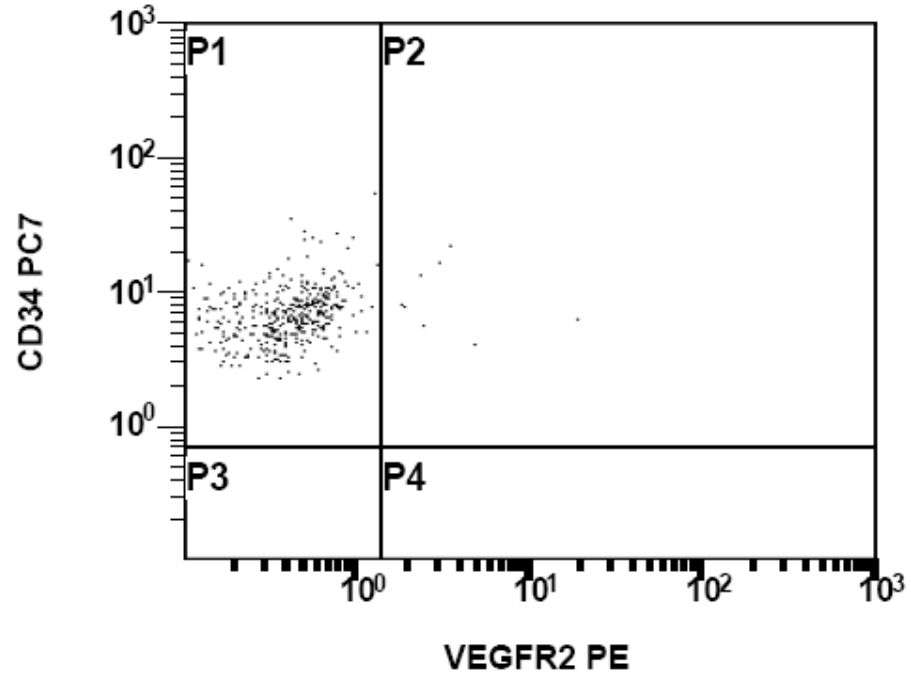


Figure A.6. Colour Dot Plot of CD34-PC7 vs. VEGFR2-PE in Lysed Whole Blood Gated on Gate E.



The cells in regions P1+P2 represent total $CD34^+$ cells, and those in P2 represent $CD34^+VEGFR2^+$/$CD34^+VEGFR2^+CD45^{dim}$ cells (figure A.6). There was no requirement for isotype due to the clear separation of single positive CD34 cells and double positive EPCs.

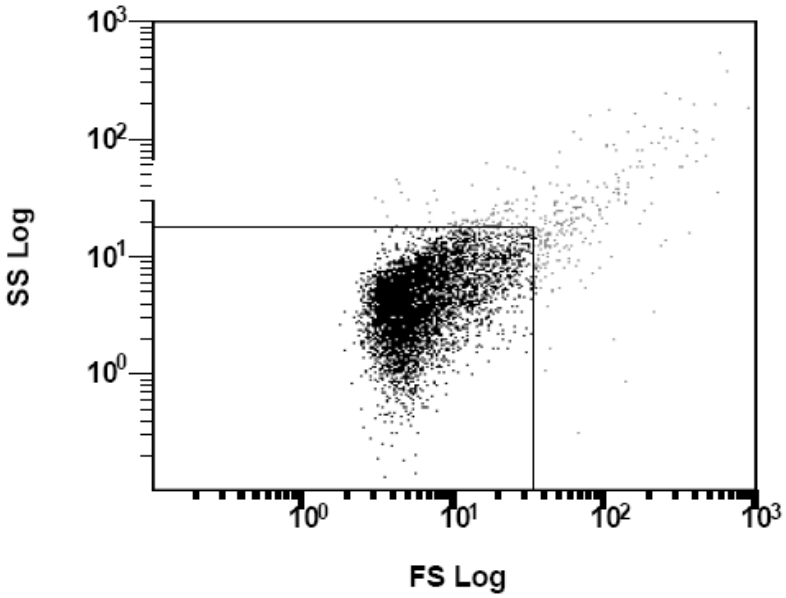
Appendix E

Cell-Derived Microparticle Analysis

Size Gate

The size of MPs measured in this study was <1.0µm. In order to measure these events it was necessary to set up a size gate of <1.0µm with FLOW-SET™ Fluorospheres (Beckman Coulter, Inc, USA) as seen in figure A.7. This gate was subsequently applied to all plots in order to eliminate larger events.

Figure A.7. Gating of <1µm Events.



Endothelial Microparticles

For the analysis of EMP, both CD62E and CD144 were used individually to define EMPs. For enumeratuion of $CD62E^+$ EMPs, a clear separation above non-specific binding was observed, and these were our true events (figure A.8). However such a clear separation was not observed for $CD144^+$ EMPs, therefore an isotype control was

applied, and events above the isotype line were taken as true $CD144^+$ events (figure A.9).

Figure A.8. Detection of $CD62E^+$ EMP.

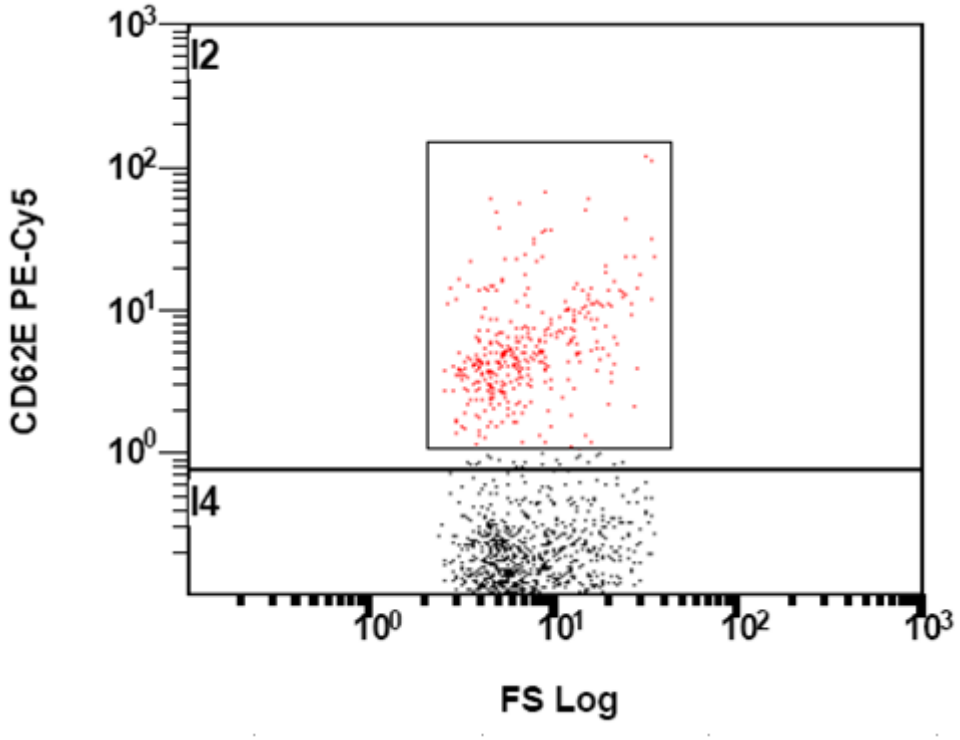
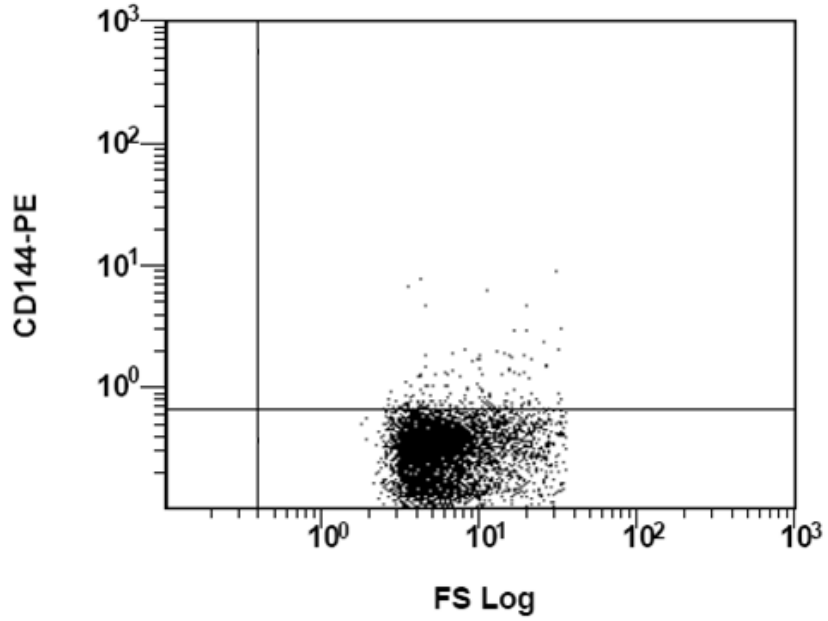


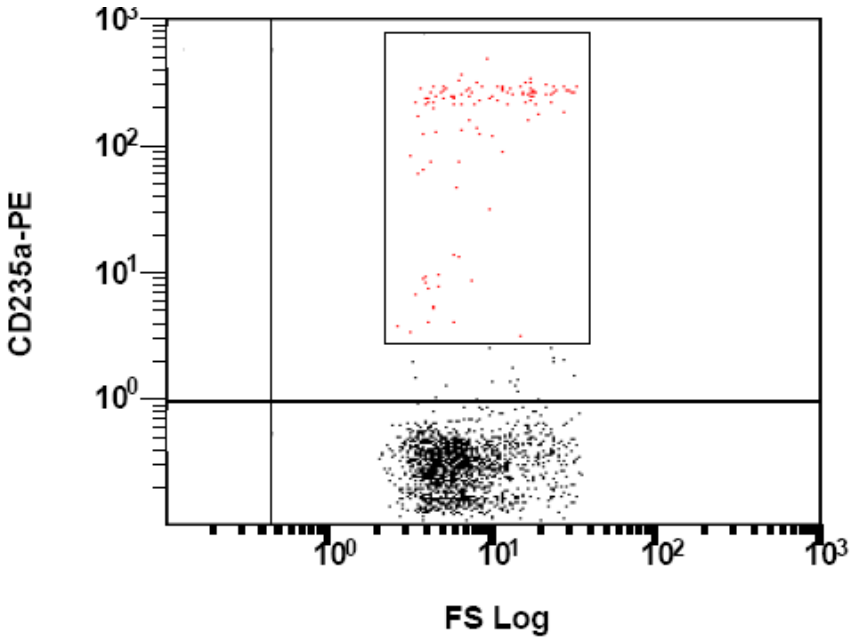
Figure A.9 Detection of CD144$^+$ EMP.



Erythrocyte-Derived Microparticles

For ErMP analysis, the RBC protein marker CD235a was used to define these events. As with the CD62E$^+$ EMPs, a clear separation above non-specific binding events was observed, thus negating the need for an isotype control (figure A.10).

Figure A.10. Detection of CD235a$^+$ ErMP.



Platelet-Derived Microparticles

PMPs were defined as CD41$^+$Annexin V$^+$ events. Firstly CD41$^+$ events were gated (figure A.11), and this gate was applied to a plot of CD41-PE against Annexin V-FITC (figure A.12). A clear population was identified and this population were the enumerated PMPs.

Figure A.11. Detection of CD41^{+} Events.

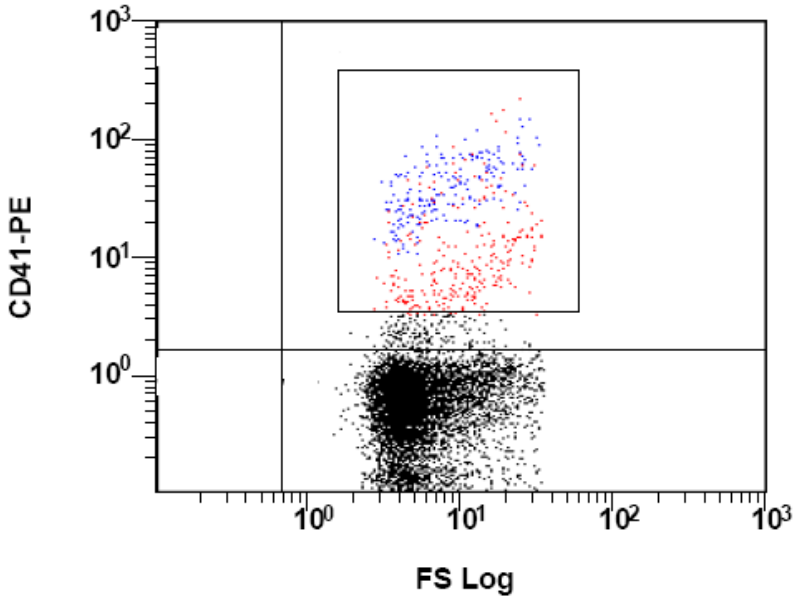
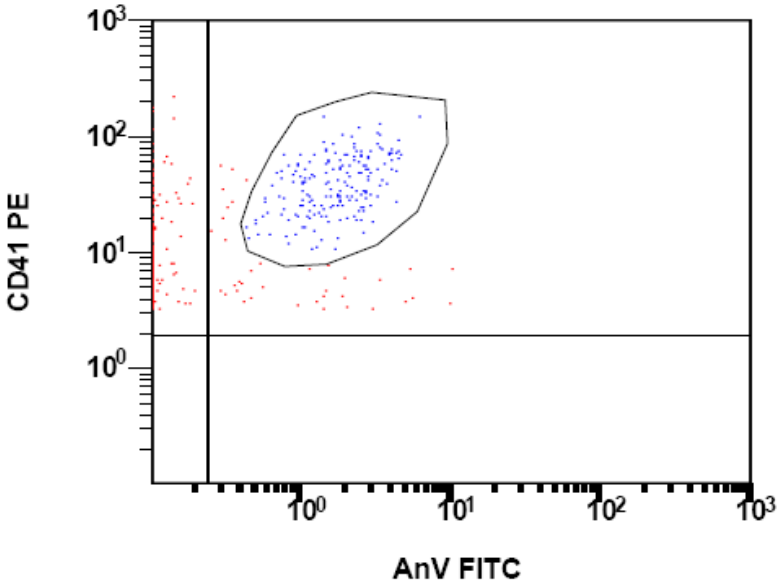


Figure A.12. CD41^{+}Annexin V^{+} PMPs.



Appendix F

EMP-bound E-selectin Procedure

Prior to analysis and preparation of the plate, 350µL aliquots of PFP was thawed and processed further at 19000g for 30 minutes at room temperature. 300µL of the supernatant removed and placed into another aliquot (for soluble E-selectin measurements; appendix G). The pellet was resuspended in 300µL PBS and further processed at 19000g for 30 minutes at room temperature. 310µL supernatant removed and discarded, and pellet was used for subsequent analysis for microparticle-bound E-selectin.

Materials Supplied:

- Read Bufer T (4X), with surfactant (Stored at room temperature)
- Blocker A kit
- MULTI-ARRAY 96-well 4 Spot E-selectin plate
- SULFO-TAG™ Anti-hE-selectin Antibody (50X)[1]
- Diluent 10
- Human E-selectin Calibrator (10µg·mL^{-1})

[1] *To be SULFO-TAG™ labelled detection antibodies may be light-sensitiv so should be kept in the dark*

Other Materials and Equipment (not supplied):

- Deionised water for diluting Read Buffer
- Phosphate Buffered Saline + 0.05% Tween-20 (PBS-T) for plate washing
- Adhesive plate seals

- Microtiter plate shaker
- Plate washer or other efficient multi-channel pipetting equipment for washing 96 well plates
- Appropriate liquid handling equipment for desired throughput that must accurately dispense 10, 25, 40 and 150µL into a 96-well microplate

Protocol:

1. Block plates with 150µL per well of Blocker A for 1 hour on a plate shaker at room temperature.

2. Wash plates 3 times with 200µL Phosphate Buffered Saline + 0.05% Tween-20 (PBS-T).

3. Add 40µL per well of Diluent 10 and 10µL per well of prepared calibrator and/or sample and incubate for 2 hours with shaking.

4. Wash plates 3 times with PBS-T.

5. Add 25µL per well of 1X Detection Antibody Reagent and incubate for 1 hour with shaking.

6. Wash plates 3 times with PBS-T.

7. Prepare SECTOR® Imager so that plate can be read immediately after Read Buffer addition.

8. Add 200µL per well of 1X Read Buffer T and read immediately with SECTOR® Imager.

Appendix G

Soluble E-selectin ELISA Procedure

Materials Supplied:

- Read Bufer T (4X), with surfactant (Stored at room temperature)
- Blocker A kit
- MULTI-ARRAY 96-well 4 Spot E-selectin plate
- SULFO-TAG™ Anti-hE-selectin Antibody (50X)[1]
- Diluent 10
- Human E-selectin Calibrator (10µg·mL^{-1})

[1] *To be SULFO-TAG™ labelled detection antibodies may be light-sensitiv so should be kept in the dark*

Other Materials and Equipment (not supplied):

- Deionised water for diluting Read Buffer
- Phosphate Buffered Saline + 0.05% Tween-20 (PBS-T) for plate washing
- Adhesive plate seals
- Microtiter plate shaker
- Plate washer or other efficient multi-channel pipetting equipment for washing 96 well plates
- Appropriate liquid handling equipment for desired throughput that must accurately dispense 10, 25, 40 and 150µL into a 96-well microplate

Protocol:

1. Block plates with 150µL per well of Blocker A for 1 hour on a plate shaker at room temperature.

2. Wash plates 3 times with 200µL Phosphate Buffered Saline + 0.05% Tween-20 (PBS-T).

3. Add 40µL per well of Diluent 10 and 10µL per well of prepared calibrator and/or sample and incubate for 2 hours with shaking.

4. Wash plates 3 times with PBS-T.

5. Add 25µL per well of 1X Detection Antibody Reagent and incubate for 1 hour with shaking.

6. Wash plates 3 times with PBS-T.

7. Prepare SECTOR® Imager so that plate can be read immediately after Read Buffer addition.

8. Add 200µL per well of 1X Read Buffer T and read immediately with SECTOR® Imager.

Appendix H

Vascular Endothelial Growth Factor (VEGF) Electrochemiluminescence Assay Procedure

Materials Supplied:

- Read Bufer T (4X), with surfactant (Stored at room temperature)
- Blocker C
- MULTI-ARRAY 96-well Small Spot VEGF plate(s)
- SULFO-TAG™ Anti-h-VEGF Antibody (100X)[1]
- Diluent 7
- Diluent 8
- Diluent 9
- Human VEGF Calibrator ($1\mu g \cdot mL^{-1}$)

[1] *To be SULFO-TAG™ labelled detection antibodies may be light-sensitiv so should be kept in the dark*

Other Materials and Equipment (not supplied):

- Deionised water for diluting Read Buffer
- Phosphate Buffered Saline + 0.05% Tween-20 (PBS-T) for plate washing
- Adhesive plate seals
- Microtiter plate shaker
- Plate washer or other efficient multi-channel pipetting equipment for washing 96 well plates

- Appropriate liquid handling equipment for desired throughput that must accurately dispense 25 and 150µL into a 96-well microplate

Protocol:

1. Block plates with 150µL per well of Blocker C for 1-2 hours at room temperature (or overnight at 4°C).

2. Wash plates 3 times with Phosphate Buffered Saline + 0.05% Tween-20 (PBS-T).

3. Add 25µL per well of Diluent 7 and 25µL per well of prepared calibrator and/or sample and incubate for 2 hous with shaking.

4. Wash plates 3 times with PBS-T.

5. Add 25µL per well of 1X Detection Antibody Reagent and incubate for 2 hours with shaking.

6. Wash plates 3 times with PBS-T.

7. Prepare SECTOR® Imager so that plate can be read immediately after Read Buffer addition.

8. Add 150µL per well of 1X Read Buffer T and read immediately with SECTOR® Imager.

Appendix I

Granulocyte-Colony Stimulating Factor (G-CSF) Electrochemiluminescence Assay Procedure

Materials Supplied:

- Read Bufer T (4X), with surfactant (Stored at room temperature)
- MULTI-ARRAY 96-Well Small Spot G-CSF plate(s)
- SULFO-TAG™ Anti-human-G-CSF Antibody (50X)[1]
- Diluent 2
- Diluent 3
- Human G-CSF Calibrator ($1\mu g \cdot mL^{-1}$)

[1] *To be SULFO-TAG™ labelled detection antibodies may be light-sensitiv so should be kept in the dark*

Other Materials and Equipment (not supplied):

- Deionised water for diluting Read Buffer
- Phosphate Buffered Saline + 0.05% Tween-20 (PBS-T) for plate washing
- Adhesive plate seals
- Microtiter plate shaker
- Plate washer or other efficient multi-channel pipetting equipment for washing 96 well plates
- Appropriate liquid handling equipment for desired throughput that must accurately dispense 25 and 150µL into a 96-well microplate

Protocol:

1. Dispense 25µL per well of Diluent 2. Seal plate and incubate for 30 minutes with vigorous shaking (300-1000rpm) at room temperature.
2. Add 25µL per well of Calibrator and/or sample and incubate for 2 hours with shaking.
3. Wash plates 3 times with PBS-T.
4. Add 25µL per well of 1X Detection Antibody Reagent and incubate for 2 hours with shaking.
5. Wash plates 3 times with PBS-T.
6. Prepare SECTOR® Imager so that plate can be read immediately after Read Buffer addition.
7. Add 150µL per well of 2X Read Buffer T and read immediately with SECTOR® Imager.

Appendix J

Waterford Institute *of* Technology
INSTITIÚID TEICNEOLAÍOCHTA PHORT LÁIRGE

RESEARCH - INFORMED CONSENT FORM

I. Project Title:

Influence of acute exercise on circulating microparticles and endothelial progenitor cells in trained and untrained men

II. Introduction to this study:

The endothelium makes up the inner layer of all blood vessels and is now know to perform a range of crucial functions related to cardiovascular health. There is considerable interest amongst scientists in endothelial microparticles and endothelial progenitor cells as markers of endothelial stress and endothelial regeneration. Endothelial microparticles are fragments of endothelial cells shed into the bloodstream in response to cell stress and injury. Endothelial progenitor cells are a type of stem cell involved in the regeneration of areas of endothelial damage and the growth of new blood vessels. These novel markers provide exercise scientists with a means of studying the effects of exercise on the endothelium (normally inaccessible) via a simple blood sample.

III. I am being asked to participate in this research study. The study has the following purposes:

1. To determine the influence of different forms of exercise on circulating microparticles and endothelial progenitor cells in young men

2. To compare the exercise response in trained and untrained men

IV. This research study will take place at Waterford Institute of Technology

V. This is what will happen during the research study:

You will undertake initial measurements in the exercise laboratory that include assessment of maximum aerobic fitness capacity, maximum strength levels and percentage body fat.

You will undertake three different forms of exercise in the laboratory on three different days, an aerobic session, a sprint session and a resistance training session. Each session will last 30 - 40 minutes. A blood sample will be taken from a forearm vein immediately prior to exercise, immediately after exercise, 1 hour and 4 hours post-exercise. These samples will later be analysed for markers related to cardiovascular health. Approximately 15 mL (three teaspoonfuls) of blood will be taken during each sample.

You will be asked to refrain from exercise training and not to consume alcohol on the day prior to each exercise session. You will also be asked to follow a similar diet for one day prior to each test.

VI. There are certain risks and discomforts associated with participation in the study

1. Strenuous exercise carries with it a very small risk of heart attack. This risk only exists for a small number of individuals with pre-existing heart problems. Every effort will be made through pre-exercise screening to identify individuals with heart and other conditions that could be made worse with exercise. A defibrillator is always on site in the laboratory.

2. Fatigue will be experienced during the exercise sessions. In addition, muscle soreness may be experienced for a day or two after exercise, particularly after the resistance training session.

3. A small amount of localised bruising can occur after a blood sample is taken. Every effort will be made to avoid this by the individual taking the blood sample.

VI. My confidentiality will be guarded:

Waterford Institute of Technology will protect all the information about me and my part in this study. My identity or personal information, will not be revealed, published or used in future studies. The study findings will form the basis for preparation of a postgraduate thesis, academic publications, conference papers and other scientific publications.

VII. If I have questions about the research project, I am free to call Dr. Michael Harrison at telephone no. 051-302161:

VIII. Taking part in this study is my decision.

If I do agree to take part in the study, I may withdraw at any point. There will be no penalty if I withdraw before I have completed all stages of the study. In the case of WIT students, academic grades and progress will not be affected in any way. However, once I have completed the study I will not be allowed to have my personal information and results removed from the database.

IX. Signature:

I have read and understood the information in this form. My questions and concerns have been answered by the researchers, and I have a copy of this consent form. Therefore, I consent to take part in this research project entitled: *"Influence of acute exercise on circulating microparticles and endothelial progenitor cells in trained and untrained men"*

Signed: _____

Date: _____

Witness:_____

Signature