Circulating Microvesicles:
Responses to Exercise and Heat Stress, and their Impact upon Human Endothelial Cells

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Abstract

Cell-derived microvesicles (MVs) are naturally released into the human circulation and an increase in the concentration of certain MV populations have been observed after exercise. However, the MV appearance dynamics, the exercise-related stimuli that induce their formation and physiological relevance are poorly understood. Hence, the overall objectives of this thesis were to: 1) characterise the circulating platelet (PMV) and endothelial-derived MVs (EMVs) responses during exercise and recovery, as well as their arteriovenous dynamics, 2) investigate the potential role of haemodynamic forces on MVs formation in vivo by vascular shear stress manipulations, and 3) explore the putative proliferative, chemotactic and angiogenic potential of exercise-derived MVs upon human vascular endothelial cells in vitro. Chapter 5 of this thesis describes the time-course of MV appearance in response to prolonged cycling, and demonstrates that intravascular [PMV] increases during and after exercise performed in the heavy intensity domain, whereas [EMV] remains unaltered. Moreover, [PMV] during exercise was related to estimates of vascular shear stress and plasma noradrenaline levels. Results from chapter 6 revealed that PMVs increased in the arterial circulation during passive heat stress, and in the arterial as well as venous circulation during short duration very heavy exercise engaging either a large or small muscle mass. The increases in [PMV] were not directly linked to local changes in vascular shear stress through heat stress and exercise, indicating a systemic PMV response. Finally, chapter 7 revealed that exercise-derived MVs supported endothelial proliferation and migration, while displaying pro-angiogenic potential in vitro. In conclusion, results of this thesis provide original information about MV dynamics, by demonstrating that PMV increase systemically in the circulation not only after but during exercise involving a small and large muscle mass. This MV response seems to be modulated by exercise intensity, and is only partially linked to levels of vascular shear stress. Moreover, circulating MVs produced during exercise present stimulatory angiogenic and mitogenic effects upon endothelial cells in vitro, suggesting a novel potential link between vascular adaptation and exercise training.

Acknowledgements

My greatest thanks, however, goes to my beloved wife and high school sweetheart, Ana, who has always supported my decisions no matter what, and has given up so much, so I could follow my own dreams. You are an example to me and your smile makes every sunrise become a day worth living for.

Finally, my sincere gratitude to the participants who made this thesis possible and to the Brazilian government for all of the investment put on me and on my colleagues around the world. Investment in education may not bring immediate outcomes, but only through it we will be able create a better future and become a more egalitarian society in the long run.

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List of Abbreviations

a-v = arteriovenous

BA = brachial artery

B-mode = brightness mode

CAD = coronary artery disease

CON = resting control trial

BF = blood flow

CRP = C-reactive protein

eNOS = endothelial nitric oxide synthase

EDTA = ethylenediaminetetraacetic acid

EDV = end diastolic volume

ELISA = enzyme-linked immunosorbent assay

M199 = medium 199

EMV = endothelial microvesicle

ESV = end systolic volume

exMVs = circulating microvesicles during exercise

FA = common femoral artery

Fc = fragment crystallisable

FBS = foetal bovine serum

FSC = forward scatter

Hb = haemoglobin

Hct = haematocrit

HI = heavy intensity trial

HR = heart rate

HUVEC = human umbilical vein endothelial cell

IL = interleukin

MAP = mean arterial pressure

MI = moderate intensity trial

MTT = thiazol blue tetrazolium bromide

MV = microvesicle

NO = nitric oxide

PMV = platelet microvesicle

PPO = peak power output

PPP = platelet-poor plasma

PS = phosphatidylserine

$\dot{Q}$ = cardiac output

rMVs = circulating microvesicles at rest

rpm = revolutions per minute

SD = standard deviation

SEM = standard error of the mean

SR = shear rate

SSC = side scatter

SV = stroke volume

TNF-α = tumor necrosis factor alpha

VEGF = vascular endothelial growth factor

$\dot{V}CO_2$ = carbon dioxide production

$\dot{V}O_2$ = oxygen uptake

$\dot{V}O_2max$ = maximal oxygen uptake

Chapter 1 - Introduction

1.1. Study background

In recent years, great interest has been placed on understanding the relevance of cell-derived microvesicles (MVs) in health and disease. The interchangeable terms "microvesicle" and "microparticle" refer to biologically active subcellular packages shed by the plasma membrane of various cells (Jimenez *et al.*, 2003; Shantsila *et al.*, 2010), and were originally observed as by-products of platelets in the late 60's (Wolf, 1967). Contemporary evidence indicates that several pathological conditions, including cardiovascular disorders, are linked with increased levels of circulating MVs (Shantsila *et al.*, 2010), and disturbed levels of these tiny vesicles may even impair endothelial function (Boulanger *et al.*, 2001; Vanwijk *et al.*, 2002). Certain MVs, however, have been reported to increase within the intravascular space after exercise, suggesting that variations in the concentration of these extracellular vesicles may be a necessary physiological response.

The rise in blood concentrations of MVs have been primarily explored during the recovery period, with several authors reporting an increase in certain populations of MVs at some point after the exercise bout (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Lansford *et al.*, 2015). Identifying the underlying mechanisms leading to acute adjustments to exercise is an important step in understanding the physiological effects of exercise, but currently the influence of different variables, such as exercise intensity and volume, have been overlooked. Since studies primarily focused on changes in MV concentration during recovery, the MV responses during exercise are also unknown. Moreover, our understanding of acute exercise bouts on intravascular MVs is restricted to data obtained from the venous circulation draining non-exercising limbs, and information about stimuli leading to their release remains sparse.

Increased physical activity levels have been known for sometime to protect against cardiovascular diseases by improving the profile of blood lipids, inflammatory factors, and arterial pressure, but amelioration of these and additional traditional risk factors with exercise seems to explain only ~50-60% of its protective effect against cardiovascular diseases (Mora *et al.*, 2007). Part of

the beneficial cardiovascular effects caused by chronic exercise (*i.e.* training) may be a consequence of enhanced endothelial function (Jensen-Urstad *et al.*, 1999; DeSouza *et al.*, 2000; Tanaka *et al.*, 2000; Walsh *et al.*, 2003), which is thought to occur in response to repetitive increases in vascular shear stress (Credeur *et al.*, 2010; Tinken *et al.*, 2010; Birk *et al.*, 2012), but other mechanisms may also act in synergy to bring about endothelial adaptations (Padilla *et al.*, 2011a). In this context, *in vitro* work outside the area of exercise indicates that certain MVs found in the circulation can protect endothelial cells (Kim *et al.*, 2004). Hence, it seems possible that exercise-derived circulating MVs may interact with endothelial cells and serve as a circulating messenger involved in the translation of exercise stress upon vascular adaptations.

Therefore, to further the present knowledge and understanding on how intravascular MVs respond to exercise and interact with the surrounding milieu, this thesis aims to explore the dynamics MV appearance in the circulation during and after moderate and heavy exercise, investigate potential mechanisms linked to their formation in response to exercise *in vivo*, and look at potential effects of exercise-derived MVs upon endothelial cell activity *in vitro*. The next chapter presents a general overview of the literature related to MVs, exercise and the vascular endothelium, followed by the thesis aims and hypotheses and the general methods used in the experimental chapters. Finally, results derived from *in vivo* (chapter 5 and 6) and *in vitro* (chapter 7) experiments will be presented, followed by the overall discussion and conclusions.

Chapter 2 - Literature Review

2.1. Circulating microvesicles

The history of microvesicle research dates back to 1967 when Peter Wolf first identified subcellular coagulant elements derived from platelets, which he called "platelet dust" (Wolf, 1967). These platelet products, later named MVs, displayed significant coagulant activity, inducing clot formation even in platelet free plasma. At the beginning of the next decade platelet membrane vesiculation after incubation with thrombin was imaged by electron microscopy (Webber & Johnson, 1970), providing some of the first images of MVs (Hargett & Bauer, 2013). Over the following years MVs were quantified in various biological fluids by different techniques including enzyme-linked immunosorbent assay (ELISA), nanoparticle tracking analysis, flow cytometry, with the recent advent of image flow cytometers enabling scientists not only quantify the number of MV, but also visualise each of them individually.

Today it is recognised that altered blood MV levels are associated with unhealthy or pathological conditions, with augmented levels reported in obesity (Esposito *et al.*, 2006; Dimassi *et al.*, 2016), dyslipidaemia (Amabile *et al.*, 2014), metabolic syndrome (Amabile *et al.*, 2014), and coronary artery disease (CAD) (Bernal-Mizrachi *et al.*, 2003) to list a few conditions. In addition, some MV populations correlate positively with poor vascular outcomes, such as endothelial dysfunction (Esposito *et al.*, 2006) and increased arterial stiffness (Wang *et al.*, 2007a), indicating a role of extracellular vesicles in the pathophysiology of cardiovascular diseases. Furthermore, abnormally reduced MV concentrations have been observed to be detrimental. For example, a low blood MV concentration is believed to be one of the underlying mechanisms involved in Scott syndrome, a rare haemorrhagic disorder (Morel *et al.*, 2011); whereas ageing, an independent risk factor for cardiovascular disease, has been linked to a reduced basal endothelial microvesicle (EMV) concentration (Forest *et al.*, 2010).

Based on the above mentioned research, it is reasonable to believe that MVs concentrations have a physiological functional range that helps maintain homeostasis, outside which they may induce a pathological phenotype. This concept, however, may be too simplistic to explain the multifaceted roles these

subcellular vesicles play, as they have also been reported to promote a dose-dependent protective phenotype in cultured endothelial cells (Kim *et al.*, 2004), and have been speculated to increase in the circulation as a compensatory mechanism under certain conditions (Dimassi *et al.*, 2016). These data associated with the fact that physical exercise (which brings about well-known benefits for the vascular system) can stimulate a temporary appearance of MVs in the circulation during recovery. This implies that these vesicles may play varied roles in the cardiovascular system. In this chapter, a review of the current literature about the interplay between MVs, the vascular endothelium and exercise, will be presented in order to provide the rationale for the studies of this thesis.

2.1.1. *Quantification and phenotyping of microvesicles*

By standard definition, MVs are anucleated cell-derived particles ranging between 0.1 to 1 µm in diameter with no synthetic capabilities (Freyssinet, 2005; Hargett & Bauer, 2013). Although some size overlap exists, extracellular vesicles smaller than 0.1 µm may be considered exosomes, while those larger than 1 µm fall within the group termed apoptotic bodies, both of which differ from MV in their formation process and function (Curtis *et al.*, 2013). The current understanding is that MVs may serve as biomarkers of cellular state since they carry parent-cell specific proteins which may change according to stimulatory conditions (Jimenez *et al.*, 2003).

Studies aiming to investigate cell-derived MVs have applied a variety of techniques. Studies employing electron microscopy for MV visualisation were very useful in characterising their release (Webber & Johnson, 1970; Cantaluppi *et al.*, 2012) (Figure 2-1). However, the primarily qualitative and time consuming nature of this technique forced researchers to rely on different methods for high throughput quantitative measurements. To overcome shortcomings related to the clinical quantification of MVs, researchers have frequently opted for flow cytometry. This technique has become the most common method for MV quantification, due to its rapid processing, enabling researchers to extract a

large amount of information from a single sample. Flow cytometry, however, is not without limitations and is beset with sensitivity issues since traditional cytometers frequently demonstrate a limit of detection for single calibration beads (that have greater refractive index than MVs) between 200-300 nm in diameter, and smaller vesicles can be misidentified as single events (van der Pol *et al.*, 2012). This gave rise to the "iceberg" hypothesis, which suggests that MV quantification is underestimated by traditional cytometers (Harrison & Gardiner, 2012). Fortunately, technological progress in this field has facilitated the commercialisation of cytometers with improved spatial resolution, and enabled the development of image flow cytometry technology. The sole commercial devise is marketed as the ImageStream^X® by Amnis Corporation, and combines the throughput of flow cytometry with the imaging capability of microscopy. A recent publication by Headland *et al.* (2014) reported a striking capacity of the ImageStream^X® Mark II in identifying events as small as 20 nm, when side scatter (SSC) signals were analysed combined with 60x camera magnification. If this applies to biological fluids, the quantification events within the whole size spectrum of MVs would be possible, but this possibility still demands further confirmatory data.
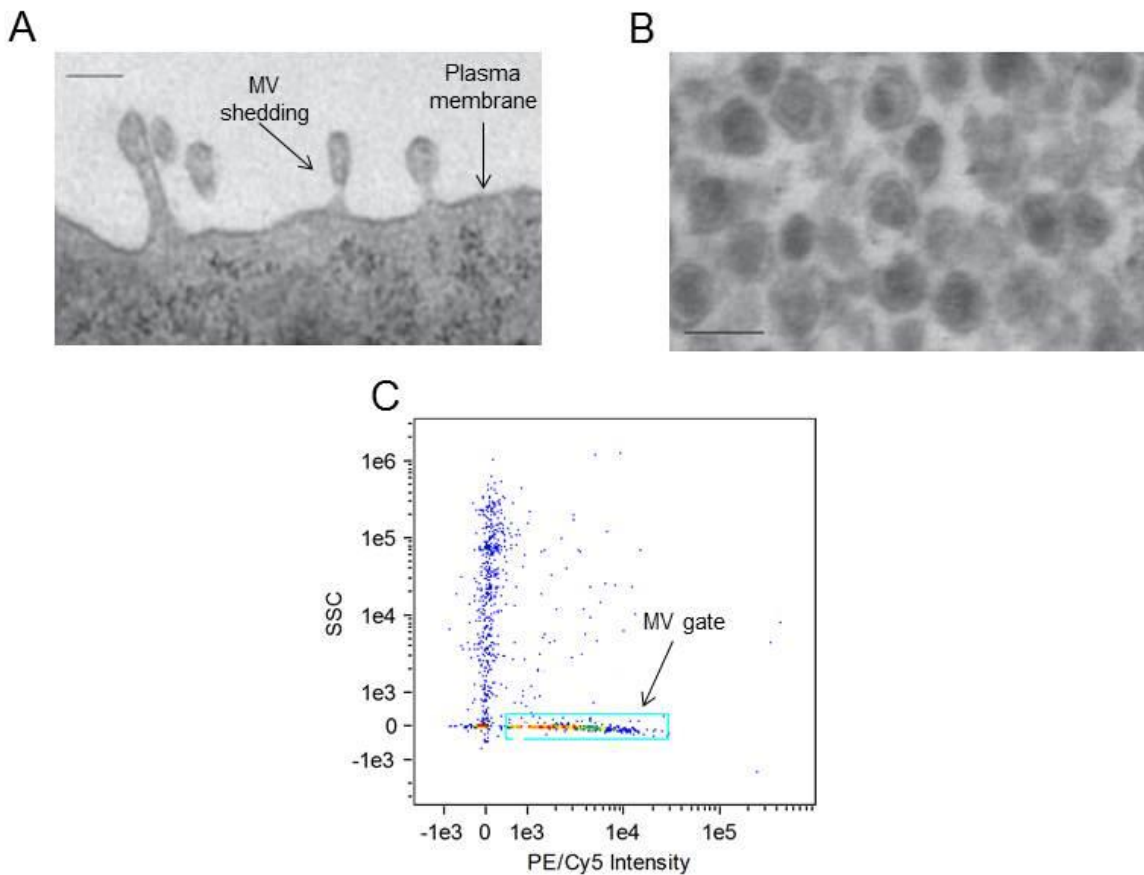
Figure 2-1. Example of transmission electron microscopy images of cell-derived microvesicle (MV) (A and B), and of image flow cytometric MV gating (C). Cell plasma membrane reorganisation results in shedding of microvesicles (A), which are round-shaped in suspension (B). A and B were adapted from other authors (Cantaluppi *et al.*, 2012)

The expression of unique antigens on the surface of MVs permits the determination of their cell source. For example, platelet derived microvesicles (PMV) can be identified by the presence of platelet proteins such as glycoprotein IIb (CD41) or glycoprotein Ibα (CD42b) (Shantsila *et al.*, 2010; Headland *et al.*, 2014). Those of endothelial origin do not express exclusive platelet antigens but may carry platelet-endothelial cell adhesion molecule (CD31), or E-selectin (CD62E) when shed during apoptotic or activation processes, respectively (Jimenez *et al.*, 2003); whereas monocyte microvesicles have been identified by the expression of the lipopolysaccharide-

receptor (Sossdorf *et al.*, 2011; Headland *et al.*, 2014). Some early approaches in the identification of MVs limited phenotyping to vesicles that expressed phosphatidylserine (PS) (Sossdorf *et al.*, 2010, 2011), owing to the concept that MV formation always involved an inversion of the plasma membrane structure with negatively charged phospholipids, including PS, apparent on the outer leaflet of the membrane. Recent data, however, challenges the definition that MVs must present anionic phospholipids, since almost 80% of MVs derived from unstimulated platelets have been shown to not stain positively for annexin-V (Connor *et al.*, 2010). Hence, the quantification of MVs in some more recent publications has been based exclusively on the expression of cell-derived antigens (Guiraud *et al.*, 2013; Lansford *et al.*, 2015), with annexin-V^{+} MVs suggested to reflect a specific pro-coagulant subpopulation (Connor *et al.*, 2010).

Several other methods for extracellular vesicle detection exist, including Raman spectroscopy, and the recently developed nanoparticle tracking analysis (van der Pol *et al.*, 2014b). The antigenic MV properties have also allowed the adaptation of antibody capture assays to immobilise and quantify these extracellular vesicles. Researchers have been successful in quantifying certain circulating MV populations using an ELISA approach (Curtis *et al.*, 2009; Maruyama *et al.*, 2012), which is promising because it may enable the determination of MVs routinely in more clinical settings. Nevertheless, it is most likely that flow cytometry will remain the dominant research technique for circulating MV quantification in the upcoming years, due to its already well established protocols and relative simplicity.

2.1.2. Mechanisms of microvesicle formation

The production of MVs is initiated by a combination of orchestrated events. It has been recognised that, under stimulation of a myriad of agonists (Table 2-1) human cells undertake recruitment of sequential pathways generally resulting in cytoskeleton proteolysis and cell shrinkage, leading to the blebbing of MVs (Chang *et al.*, 1993; Morel *et al.*, 2011). Since extracellular vesicles of

platelet origin were the first identified and constitute the predominant subpopulation in human plasma, most early investigations regarding their underlying mechanisms of generation were based on PMVs, nonetheless more recent work has focused on the agonists responsible for the formation of EMVs.

Table 2-1. Example of stimuli related to microvesicle formation

Stimulus	Platelet Microvesicle	Endothelial Microvesicle	Reference
High shear stress	+	-	Miyazaki *et al.* (1996), Reininger *et al.* (2006), Vion *et al.* (2013b)
Low shear stress	-	+	Vion *et al.* (2013b)
Wall stretching	?	+	Vion *et al.* (2013a)
TNF-α	?	+	Jimenez *et al.* (2003)
IL-1α	?	+	Abid Hussein *et al.* (2008)
IL-6	+	?	Nomura *et al.* (2000)
C-reactive protein	?	+	Wang *et al.* (2007)
Angiotensin II	?	+	Burger et al. (2011)
Thrombin	+	+	Brill *et al.* (2005) Sapet *et al.* (2006)
Noradrenaline	+	?	Tschuor *et al.* (2008)

TNF-α, tumor necrosis factor α; IL-1α, interleukin-1α; IL-6, interleukin-6; + increase; - no change; ? unknown.

An intact platelet membrane presents asymmetric phospholipid distribution with negatively charged varieties, such as PS, located on the inner

leaflet, whereas neutral phospholipids are found on the outer membrane leaflet. Amongst platelets, PS serves as a catalytic site for thrombin generation, which explains in part the potent pro-coagulant activity of MVs expressing PS (Abid Hussein *et al.*, 2008; Connor *et al.*, 2010). Hence, to maintain proper cellular function and to regulate vascular haemostasis, this irregular membrane distribution is required and can be altered when necessary. The abundance of PS on the cytosolic leaflet of the cell membrane is regulated by the aminophospholipid translocases flippase and floppase (Chang *et al.*, 1993). Flippase are ATP-dependent proteins that transport PS to the inner membrane, whereas the floppase can disturb membrane asymmetry by outward translocation of this phospholipid. During cellular activation or apoptosis, a randomization in membrane phospholipid content occurs, resulting in an increased appearance of PS on the outer membrane, which coupled with cytoskeleton reorganisation through the activation of intracellular pathways, induce membrane remodelling and shedding of newly formed MVs that may express PS on their surface (Morel *et al.*, 2011).

The presence of PS on the MV surface can be detected by the binding of annexin-V, and this feature was initially considered an essential amongst MV populations, leading to the concepts that 1) their formation depended on the loss of cellular membrane phospholipid asymmetry with PS extrusion, and 2) that all MVs exposed this negatively charged phospholipid. However, PS exposure alone does not necessarily initiate vesiculation in platelets (Dachary-Prigent *et al.*, 1995), indicating that this event is linked to, but not a cause of MV formation. The intra-cellular pathways that eventually result in vesiculation are actually multifaceted and depend on the stimulatory agonist and cell type (Figure 2-2). The activation of calcium-dependent proteins was initially suggested as a central mechanism in platelet vesiculation, as Chang *et al.* (1993) observed an increase in PS translocation, and MV release from platelets when their cytoplasmatic Ca^{2+} concentrations were increased through agonist stimulation. Calcium chelating agents eliminated this agonist-induced PS migration to the outer membrane and PMV formation, reinforcing the involvement of a Ca^{2+}-dependent pathway in these processes. This particular vesiculation pathway may be governed by the activation of Ca^{2+}-dependent

proteases with subsequent cytoskeleton reorganisation. For example, Dachary-Pringent *et al.* (1995) confirmed that Ca^{2+}-ATPase inhibition, which elevates intracellular Ca^{2+}, led to subsequent MV release from platelets, with specific blocking of 97 kDa Ca^{2+}-ATPase protein blunting both calpain activity and platelet vesiculation. Furthermore when direct calpain inhibitors were applied a marked reduction PMV formation was observed (Fox et al., 1991).
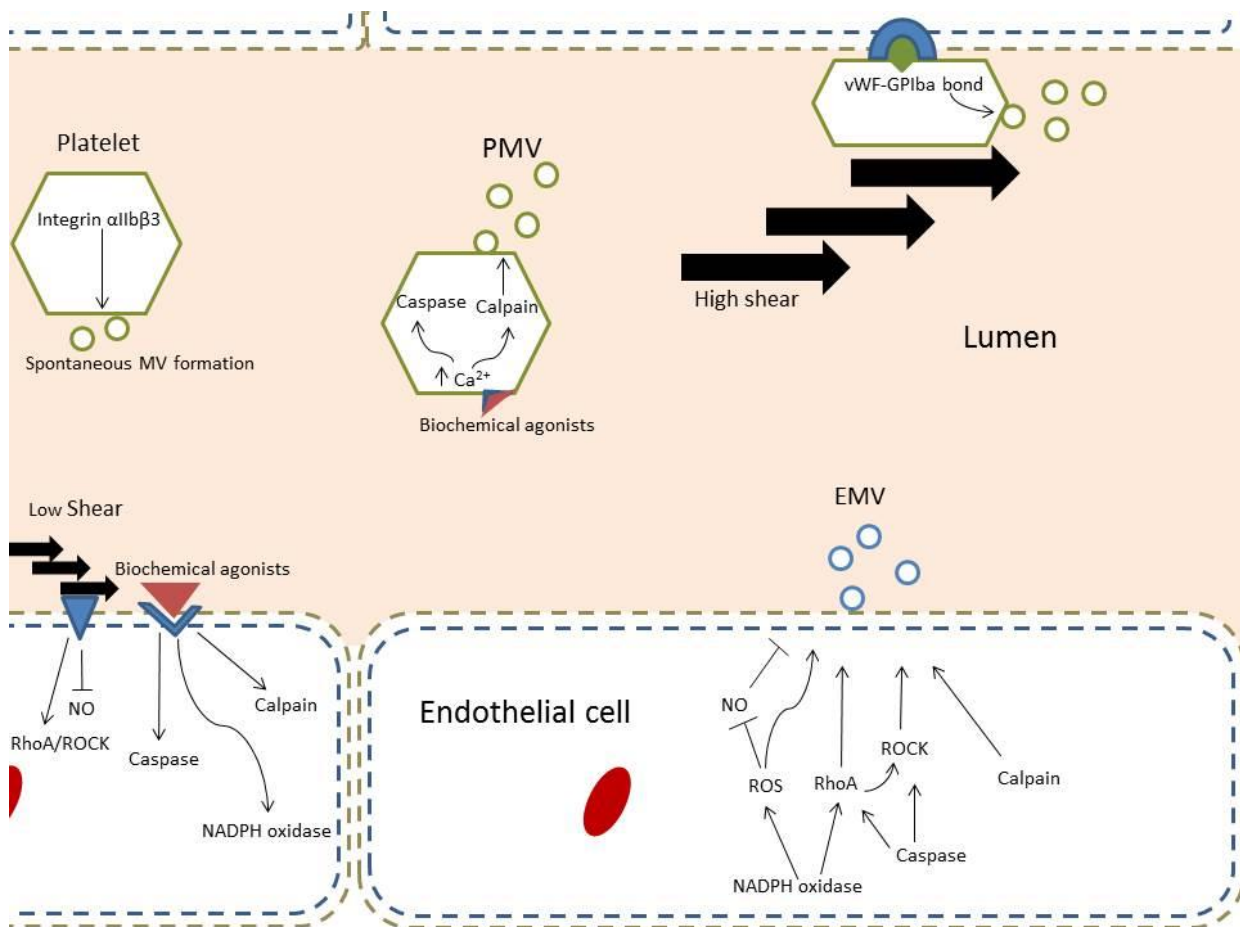
Figure 2-2. Example of agonists and pathways related to platelet microvesicle (PMV) and endothelial microvesicle (EMV) formation. In endothelial cells (bottom), low shear stress may activate the Ros homolog gene family, member A (RhoA)/Rho-associated, coiled-coil containing protein kinase (ROCK) pathway, and inhibit nitric oxide (NO) production, while some biochemical agents such as cytokines and hormones have been identified to activate Ca^{2+}-dependent proteases, and trigger reactive oxygen species production through membrane nicotinamide adinine dinucleotide phosphate-oxidase (NADPH oxidase), and an increase in all of these, but NO, pathways have been implicated in EMV formation (Sapet *et al.*, 2006; Vion *et al.*, 2013b). Spontaneous shedding of MV by non-activated platelets is dependent on function of integrin αIIbβ3, while both shear stress and biochemical agents can induce PMV formation through the caspase/calpain pathways and platelet arrest with by immobilised von Willebrand factor-glycoprotein Ibα bond, respectively (Fox *et al.*, 1991; Chang *et al.*, 1993; Cauwenberghs *et al.*, 2006; Reininger *et al.*, 2006).

Other studies have identified additional agonist-dependent pathways in platelets and other cell types (Cauwenberghs *et al.*, 2006; Sapet *et al.*, 2006). Examining human microvascular endothelial cells, Sapet *et al.* (2006) have shown that caspase-2 blocking can prevent the release of EMV after thrombin stimulation, with no effect on basal EMV production, and Cauwenberghs *et al.* (2006) documented a role of integrin αIIbβ3 activity as a calcium independent-stimulus of MV release from unstimulated platelets. These authors reported that the application of calcium chelator agents or calpain inhibitors had only minor effects on basal PMV formation, while integrin αIIbβ3 blockade abolished spontaneous shedding of MV from non-active platelets. The fact that this integrin can also stimulate calpain activity suggests it has alternative calpain-dependent and independent effects leading to MV release.

Haemodynamic forces are also known to influence the release of MVs, with elevated shear stress shown to act as an agonist inducing platelet vesiculation (Miyazaki *et al.*, 1996; Reininger *et al.*, 2006). As described by Reininger *et al.* (2006), stimulation with increasing shear forces caused *ex vivo* platelet attachment and immobilisation with von Willebrand factor. There was also successive tethering to membranes attributable to hydrodynamic drag, and PMV formation in a von Willebrand factor-glycoprotein Ibα bond-dependent pathway.

The potential for vascular shear stress to modulate endothelial cell function and vascular health is well known, and EMV shedding seems to reflect the reaction of endothelial cells to shear forces *in vitro*. Studying human umbilical vein endothelial cells (HUVECs) under different shear forces produced by a cone-and-plate viscosimeter, Vion *et al.* (2013) observed low vesiculation when cells were exposed to high shear stress. This pattern was altered under modified settings with a drastic increase in EMV release under low shear and no shear experiments. The blocking of Rho-associated protein kinases hampered this EMV formation, showing that this pathway may be implicated in the low shear-mediated MV production from endothelial cells. These authors also observed that endothelial nitric oxide synthase (eNOS) inhibition by L-N[G]-nitroarginine methyl ester resulted in EMV production even during high shear stress, demonstrating a putative role of shear-induced NO production in blunting

the shedding of EMVs. These data agree with *in vivo* results of Jenkins *et al.* (2013) demonstrating that disturbing upstream arterial haemodynamics and shear stress by venous congestion increased the appearance of circulating EMVs. In addition, studies report an increase in blood EMVs with prolonged inactivity or bed rest (Navasiolava *et al.*, 2010; Boyle *et al.*, 2013).

In summary, the release of extracellular vesicles is achieved through different and sometimes complementary pathways. It is believed that MVs are formed through a general process involving membrane cytoskeleton reorganisation resulting in cell shrinkage, sometimes involving exposure of negatively charged membrane phospholipids, and ultimately leading to MV blebbing from the parental cell membrane. These events, however, appear to be highly dependent on the agonist and cell type investigated.

2.2. Vascular endothelium and microvesicles

2.2.1. Overview of endothelial cells

The vascular endothelium plays a major role in cardiovascular homeostasis and has been studied *in situ* for a long time, but the landmark for defining endothelial structural characteristics was the development of a method for culture of endothelial cells. According to Nathan and Jaffe (2004), many researchers have tried to culture these inner vascular cells, which were initially thought to be no more than fibroblasts by some scientists in the 1960's. However, in their two seminal works published over the following decade Jaffe and colleagues demonstrated that HUVECs could be cultivated and grown *in vitro*, presenting distinct characteristics (Jaffe *et al.*, 1973a; Jaffe *et al.*, 1973b). Unlike fibroblasts and smooth muscle cells, cultured HUVECs presented a polygonal shape, Weibel-Palade bodies, and expressed antigens of the ABH blood system of the donor (Jaffe *et al.*, 1973b). In addition, evidence was given that these cultured cells exclusively synthesised von Willebrand Factor (Jaffe *et al.*, 1973a), a claimed endothelial marker at that time (Hoyer *et al.*, 1973). These morphological and immunological criteria could differentiate the cultured cells from their previously presumed fibroblast or smooth muscle origin

(Nachman & Jaffe, 2004), opening new horizons for the study of the functions of the vascular endothelium.

Investigations characterising endothelial cells further expanded the unique knowledge of these cells in relation to vascular morphology and function. Today it is known that endothelial cells originate from haemangioblast cells and form a single layer of specialised epithelial cells that cover the innermost lining of vessels, acting as an active semi-permeable barrier between the blood and the surrounding tissues (Michiels, 2003; Aird, 2012). The mature endothelium exhibits a prominent nucleus, and has a polygonal shape in culture, even though different architectures are observed *in situ* as a consequence of its surroundings (Jaffe *et al.*, 1973b; Elgjo *et al.*, 1975). Regarding its subcellular structures, endothelial cells are usually characterised by the aforementioned Weibel-Palade bodies, and are rich in membrane invaginations named caveolae where more than 60% of eNOS has been found in rat lung microvasculature *in situ* (Rizzo *et al.*, 1998). Additionally, the vascular endothelium is abundant in organelles, such as mitochondria, Golgi complexes and both smooth and rough endoplasmatic reticula (Elgjo *et al.*, 1975), characteristics of highly metabolically active cells.

The functional endothelium produces a host of vasoactive substances that are generally divided into endothelial-derived relaxing and contracting agents. Particular interest has been placed upon the relaxing agent nitric oxide (NO), a gaseous molecule produced in endothelial cells by nitric oxide synthases and with a fundamental role in the regulation of vascular tone by decreasing vascular smooth muscle cell Ca^{2+} concentration (Blatter & Wier, 1994). This and additional endothelial messengers are also known to inhibit platelet aggregation and smooth muscle cell proliferation (Michiels, 2003), allowing the endothelium to interact with the vascular components to protect against atherosclerosis and thrombus formation. A dysfunctional endothelium, however, is believed to be exposed to increased levels of oxidative stress (Eskurza *et al.*, 2004; Donato *et al.*, 2008), expresses greater amounts of pro-inflammatory molecules (Donato *et al.*, 2008), and vasoconstricting substances such as endothelin-1 (Donato *et al.*, 2009) which, coupled with a decreased bioavailability of NO, induces a pro-atherogenic phenotype.

Hence, preserved vascular function plays a fundamental role in cardiovascular protection (Schächinger *et al.*, 2000; Inaba *et al.*, 2010). Several environmental stimuli including physical inactivity and smoking disturb endothelial function (Celermajer *et al.*, 1993; Navasiolava *et al.*, 2010; Boyle *et al.*, 2013), whereas exercise training provides a potent stimulus to counteract endothelial dysfunction (Jensen-Urstad *et al.*, 1999; DeSouza *et al.*, 2000; Tanaka *et al.*, 2000; Walsh *et al.*, 2003). Because the vascular endothelium plays a central role in the control of vascular health, it must promptly respond to alterations in the vascular milieu, including changes in MV concentrations.

2.2.2. *Microvesicle – endothelial interactions*

The location of endothelial cells, in direct exposure to blood constituents, makes the vascular endothelium a key sensor of variations in the environment, including alterations in levels of circulating MVs. The actual effects of MV upon the endothelial layer are diverse and dependent on the MV source. Circulating MVs can be seen as travelling intercellular messengers, able to reprogram target cells. Their effects can be accomplished by interacting with the recipient cell by at least one of three main ways (Figure 2-3): (1) Circulating MVs can bind to the cell membrane and interact with surface receptors; (2) can fuse with the plasma membrane with subsequent release of their cargo within the cell; or even (3) be internalised by the target cell to promote intracellular effects (Mause *et al.*, 2010). Regarding endothelial cell-microvesicle interactions, the binding of PMVs on the surface of cultured endothelia seems to be debatable (Merten *et al.*, 1999), but internalisation of MVs has been reported, by demonstrating the presence of these vesicles within the cytoplasmatic space of recipient HUVECs (Terrisse *et al.*, 2010; Dasgupta *et al.*, 2012). The treatment of PMVs with annexin-V conjugates or integrin-blocking agents blunted their uptake by HUVECs, indicating an important function of PS and platelet integrins in PMV recognition and internalisation by endothelial cells (Dasgupta *et al.*, 2012).
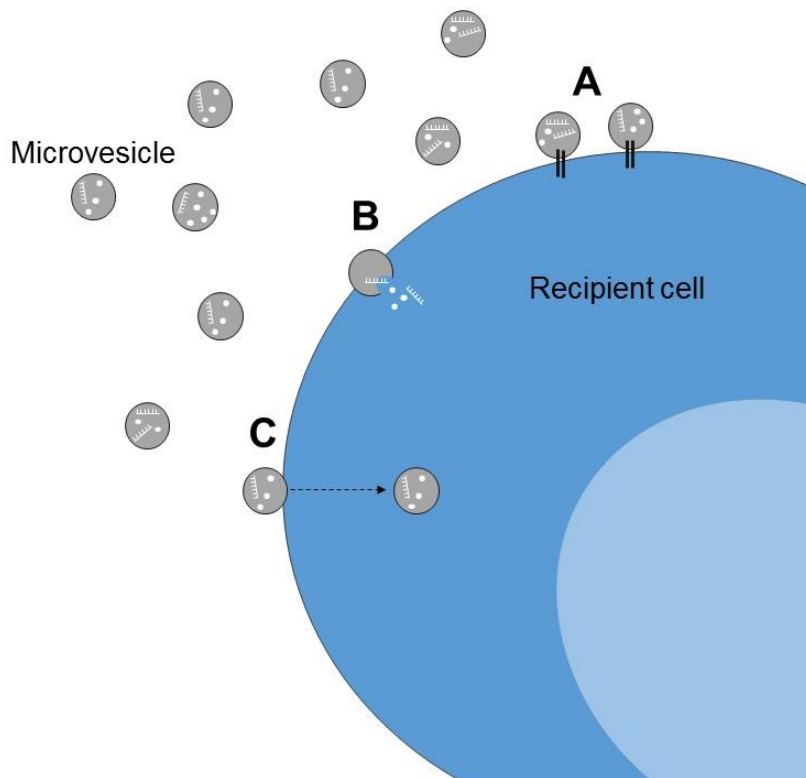
Figure 2-3. Illustration of potential microvesicle-cell interactions. Microvesicles can stimulate recipient cells by direct binding on the cell surface (A), fusing with the plasma membrane (B) or after being internalised into the cytosol (C) (Mause *et al.*, 2010).

The various effects of MVs upon recipient cells is not surprising, owing to their ability to deliver bioactive lipids, enzymes, and messenger ribonucleic acids from their parental cell (Kim *et al.*, 2004; Morel *et al.*, 2011; Horn *et al.*, 2013; Dimassi *et al.*, 2016). In relation to the vascular endothelium, it has been demonstrated that lymphocyte-derived MVs may suppress *in vivo* and *in vitro* angiogenesis by downregulating the endothelial expression of vascular endothelial growth factor (VEGF) receptors coupled with increased superoxide anion production (Yang *et al.*, 2008), and pathological levels of EMVs may have paracrine effects resulting in decreased NO bioavailability through the production of reactive oxygen species by target endothelial cells (Brodsky *et al.*, 2004). Also, EMVs produced under tumor necrosis factor alpha (TNF-α) stimulation can display pro-inflammatory potential as determined by an increased release of intercellular adhesion molecule-1 from recipient endothelial cells (Curtis *et al.*, 2009). Supporting this process, an interesting study by Boulanger *et al.* (2001) demonstrated that circulating MVs obtained from

patients with a previous myocardial infarction reduced acetylcholine-induced endothelial mediated dilation *in situ* and similar results were found with circulating MVs from pre-eclamptic women (Vanwijk *et al.*, 2002). Other manipulations involved incubating aortic rings of rodents with EMVs isolated from cell cultures obtained similar results (Brodsky *et al.*, 2004).

The above mentioned findings lead to the theory that circulating MVs may be detrimental for the vascular endothelium, but these extracellular vesicles have also been reported to bring about positive responses. For example, experimentally-induced limb ischaemia increases the concentration of MVs expressing skeletal muscle and endothelial antigens within local muscle homogenates of mice, and these MVs potentiate progenitor cell differentiation into an endothelial phenotype and subsequent angiogenesis within limbs (Leroyer *et al.*, 2009), which is relevant for understanding some of the exercise responses of interest for this thesis. MVs of platelet origins have also been linked to endothelial stimulation. Kim *et al.* (2004) demonstrated that PMVs protect HUVECs from apoptosis, and there is evidence indicating that PMVs bring about angiogenesis *in vitro* (Kim *et al.*, 2004; Brill *et al.*, 2005), and *in vivo* (Brill *et al.*, 2005). These findings are supported by results showing that plasma MVs from healthy individuals may serve as a circulatory source of active eNOS (Horn *et al.*, 2013) and that PMVs carry VEGF (Brill *et al.*, 2005), both of which are essential for proper endothelial function and the process of angiogenesis.

This apparent discrepancy between the roles of MVs may be related to the events leading to their formation under pathological and non-pathological conditions. For instance, spontaneously produced MVs found in the blood of healthy donors did not impair endothelial function in the studies by Boulanger *et al.* (2001) and Vanwijk *et al.* (2002). Likewise, EMVs released naturally by unstimulated cells were not involved in the formation of a pro-inflammatory phenotype amongst recipient endothelial cells (Curtis *et al.*, 2009). This supports the hypothesis that MV functions vary not only depending on their source, but also on their stimulus of formation, with physiological challenges probably resulting in the production of qualitatively distinct MVs compared to those formed under pathological stresses.

2.3. Exercise and microvesicles

2.3.1. Circulating microvesicle appearance with exercise

Acute exercise brings about a myriad of physiological adjustments, so that homeostasis can be maintained at a much higher metabolic demand than resting conditions. Repeated exercise over several weeks induces chronic adaptations in many physiological systems, including functional (DeSouza *et al.*, 2000; Walsh *et al.*, 2003; Rakobowchuk *et al.*, 2008; Schaun *et al.*, 2011) and structural (Tanaka *et al.*, 2000; Rakobowchuk *et al.*, 2008; Rakobowchuk *et al.*, 2013) vascular adaptations. Cellular vesiculation seems to be affected by acute exercise, with several investigations reporting increased plasma concentrations of certain MVs after exercise (Table 2-2). Sossdorf *et al.* (2010) were among the first to demonstrate that cycling stimulates the appearance of PMVs in the circulation during recovery, a finding that was later confirmed during subsequent investigations (Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Maruyama *et al.*, 2012). Other studies reported that EMVs may also be responsive to an exercise stress (Sossdorf *et al.*, 2011; Kirk *et al.*, 2013; Lansford *et al.*, 2015), although the magnitude of the response is often smaller compared to PMVs, whereas those MVs derived from other cell sources, such as erythrocytes and monocytes, remaining relatively stable (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011).

The increase in intravascular [PMV] and [EMV] after exercise, however, is not a consistent finding (Mobius-Winkler *et al.*, 2009; Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Guiraud *et al.*, 2013; Ross *et al.*, 2014; Wahl *et al.*, 2014). For example, Guiraud *et al.* (2013) reported that the plasma concentration of these MVs remained unaltered after exercise in CAD patients, but more recent work demonstrated that CAD patients may actually lack a response of MVs to dubutamine infusion (a stress test that partially mimics exercise) with an elevation in their plasma concentrations possibly being a normal physiological adjustment in otherwise healthy individuals (Augustine *et al.*, 2014). Moreover, Sossdorf *et al.* (2011) reported that elevation in EMVs observed after exercise

was limited to trained individuals, and even a decrease in this MV population has been observed after exercise (Wahl *et al.*, 2014), which is difficult to explain.

Therefore, based on the aforementioned work it seems that PMVs are the most responsive circulating MV population with currently 4 out of 5 studies involving healthy humans reporting an increase in their concentration after exercise. However, only 3 out of 8 studies under similar conditions support this finding when examining EMVs.

Table 2-2. Studies investigating shedding of microvesicles with exercise

Study	Participants	MV phenotype	Method	Blood sample timing	Exercise mode	Exercise protocol	Main results
Mobius-Winkler *et al.* (2009)	18 healthy men	CD42b⁻ MV, EMV	Flow cytometry	Before During after (until 24h)	Cycling	4h at 70% of anaerobic threshold	No change
Sossdorf *et al.* (2010)	16 healthy men	PMV, EMV, MMV	Flow cytometry	Before After: 0 min; 45 min; 2h	Cycling	1.5h at 80% of anaerobic threshold	↑ PMV at 0 min, 45 min and 2h post protocol; ↑ PMV pro-coagulant activity after exercise
Sossdorf *et al.* (2011)	8 untrained, and 8 trained men	AnnV⁺ MV, PMV, EMV, MMV	Flow cytometry	Before After: 0 min; 45 min; 2h	Cycling	1.5h at 80% of anaerobic threshold	Untrained: ↑AnnV⁺ MV and PMV until 2h after exercise; Trained: ↑ PMV, EMV and MMV at 45min; and AnnV⁺ MV until 2h after protocol
Chaar *et al.* (2011)	7 healthy men	AnnV⁺ MV, PMV, EMV, MMV, RBCMV, PMNMV	Flow cytometry	Before After: 0 min; 2h	Cycling	3 maximal ramp tests, with 10 min recovery between trials	↑ AnnV⁺ MV; PMV and PMNMV after the protocol
Mayurama *et al.* (2012)	9 men, and 9 women	PMV	ELISA	Before After: 0 min; 1h	Running	Bruce treadmill protocol until 85% of the estimated maximum heart rate	↑ PMV after the protocol
Guiraud *et al.* (2013)	19 physical active CAD male patients	EMV, PMV	Flow cytometry	Before After: 20 min; 24h; 72h	Cycling	HI interval exercise and isocaloric MI exercise (no details given about protocols)	No change
Kirk *et al.* (2013)	7 healthy men	EMV	Flow cytometry	Before After: 0 min; 1.5h; 3h	Cycling	10x15 sec sprints at 120% PPO and 45 s of active recovery under NaHCO₃ supplementation or placebo.	↑ EMV at 1.5 h compared to post 0 min

(Continue)

Ross *et al.* (2014)	13 trained male	EMV	Flow cytometry	Before After: 10 min; 2h; 24h	Resistance exercises	3 resistance exercise circuits at 15RM	No change	
Wahl *et al.* (2014)	12 male endurance athletes	PMV, EMV, MMV	Flow cytometry	Before After: 0 min; 1h; 3h	Cycling	120 min at 55% PPO; 4x4 min at 90-95% PPO with 3 min active rest, or 4x30 s all out exercise with 7.5 min active rest	Reduction in PMV, EMV and MMV 1h and 3h after protocols	
Lansford *et al.* (2015)	9 men and 9-8 women	EMV and CD34$^+$ MV	Flow cytometry	Before and within 5 min after	Cycling	Continuous exercise at 60 – 70% of $\dot{V}O_2$peak until total energy expenditure equivalent to 598 kcal (~40-60 min)	Males: ↑ EMV after the protocol Females: ↑ CD34$^+$ MV after the protocol No changes when dataset from both sexes were analysed together	

CAD, coronary artery disease; AnnV, annexin-V; MV, microvesicle; EMV, endothelial microvesicle; PMV, platelet microvesicle; MMV, monocyte microvesicle; RBCMV, red blood cell microvesicle; PMNMV, polymorphonuclear cell microvesicle; ELISA, enzyme-linked immunosorbent assay; HI, high intensity; MI, moderate intensity; 15RM, 15 repetition maximum; PPO, peak power output; $NaHCO_3$, sodium bicarbonate; $\dot{V}O_2$peak, peak oxygen uptake; ↑, increase.

2.3.2. *Circulating microvesicle responses with exercise*

The proper characterisation of a physiological phenomenon requires the description of its kinetics. However, the time-course of MV appearance in the circulation with exercise has not been systematically characterised. So far, Mobius-Winkler *et al.* (2009) were the only researchers to investigate MV shedding during exercise and reported no changes in [EMV] during 4 h of cycling and subsequent recovery. This lack of information limits our current knowledge of MV dynamics during exercise and the understanding of the underlying mechanisms of formation.

In relation to the post-exercise recovery period, 4 studies found no increase in any MV population investigated (Mobius-Winkler *et al.*, 2009; Ross, 2011; Guiraud *et al.*, 2013; Wahl *et al.*, 2014), with the remaining studies reporting augmented concentrations of at least one population of MV after exercise (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Maruyama *et al.*, 2012; Kirk *et al.*, 2013; Lansford *et al.*, 2015). The most consistent results were observed in relation to PMVs and EMVs. It seems that these two MV populations may exhibit a fast appearance rate immediately after exercise, with peak concentrations observed sometime within 2 h of the exercise session (Sossdorf *et al.*, 2010, 2011; Maruyama *et al.*, 2012). Few researchers have explored [PMV] or [EMV] responses beyond 2 h into recovery, but those who did, observed values that were lower (Wahl *et al.*, 2014) or similar to baseline at 3 h (Guiraud *et al.*, 2013), 24 h (Guiraud *et al.*, 2013; Ross *et al.*, 2014) and 72 h (Ross, 2011). Sossdorf *et al.* (2011) found that the training status of the participants may influence the response of some MV populations, however a detailed mechanistic understanding has not been provided and needs to be addressed.

Although limited information is currently available regarding the kinetics of MV formation with exercise, it seems reasonable to hypothesise based on the current literature that a progressive elevation in these tiny circulatory vesicles start to occur during exercise, peaking around 1-2 h post-exercise, and probably returning to baseline within 3 h of the session (Figure 2-4). The time-course of

circulating MVs, however, requires investigation, particularly the exercise responses and will be a major focus of this thesis.
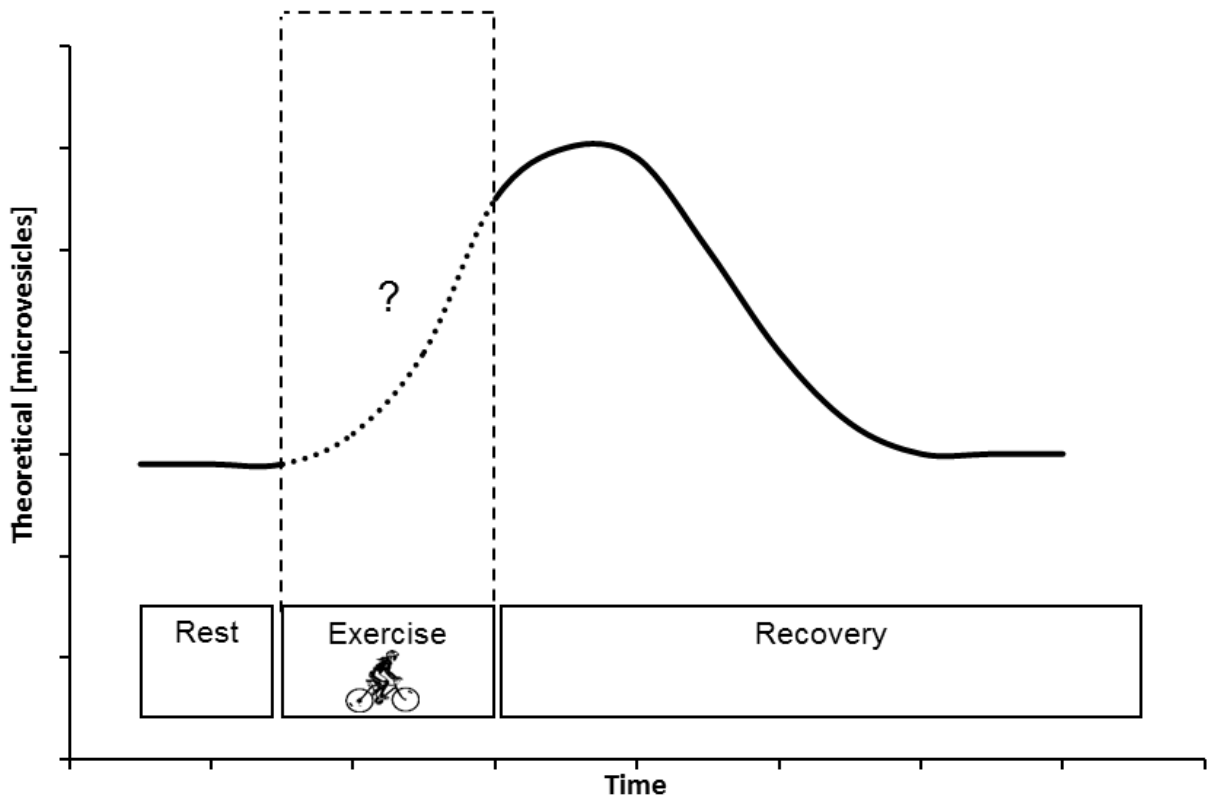
Figure 2-4. Theoretical model of microvesicle shedding kinetics with exercise. Based on the current literature, I proposed that the microvesicle response to exercise will peak within 2 h of post-exercise recovery. The time-course of appearance during exercise remains unknown.

2.3.3. Exercise intensity and microvesicles

Intensity, alongside volume, is considered one of the most important variables governing physiological responses to exercise. For a fixed volume (*e.g.* time or repetitions) higher exercise intensities require greater energy expenditure and metabolic demand, with parallel cardiorespiratory adjustments (Pritzlaff *et al.*, 2000; Lansley *et al.*, 2011). Repeated exercise sessions will eventually be translated into long-term training adaptations, and the current guidelines of the American College of Sports Medicine indicates that a minimal

exercise intensity of 45% of oxygen uptake reserve or ~45% of heart rate (HR) reserve is required to bring about improvements in maximal aerobic capacity of young healthy and relatively active individuals, whereas even lower intensities may suffice to improve physical fitness in less fit individuals (Garber *et al.*, 2011). The effect of exercise intensity on the acute release of MVs, however, has not received much attention, with only two recent studies shedding some light upon this topic.

Wahl *et al.* (2014) had cyclists/triathletes perform a high volume continuous exercise session (2 h cycling at 55% of peak power output (PPO)), and two interval exercise sessions (at submaximal or supramaximal intensities relative to PPO), and observed similar responses amongst all conditions suggesting no influence of intensity. However, this was the sole investigation in which a reduction in the concentration of MVs was observed with exercise making it difficult to interpret these findings. Guiraud *et al.* (2013) also compared the concentration of circulating MVs in response to either heavy intensity interval exercise or lower intensity continuous exercise in CAD patients and reported a lack of change after any exercise protocol. However, as mentioned earlier this population has been shown to display reduced vesiculation responses to cardiac stress tests (Augustine *et al.*, 2014) which limits the extrapolation of these results to healthy individuals. Furthermore, a drawback of these studies was that independent variables other than exercise intensity, such as different exercise:rest ratios and total exercise duration, were also different between exercise sessions. Thus, it is reasonable to conclude that these studies had a number of confounding factors that could have influenced their results.

Analysis of individual studies leads to inconsistent outcomes. While an augmentation of MV release was found in the two reports where participants performed maximal or near maximal incremental tests (Chaar *et al.*, 2011; Maruyama *et al.*, 2012), those involving submaximal prolonged exercise are contradictory (Walsh *et al.*, 2003; Mobius-Winkler *et al.*, 2009; Sossdorf *et al.*, 2010, 2011; Guiraud *et al.*, 2013). For example, Sossdorf *et al.* (2010, 2011) found shedding of MVs after 1.5 h of continuous cycling at 45% of PPO, whereas Mobius-Winkler reported no response with 4 h at 70% of the anaerobic

threshold, and a session performed at 55% PPO was reported to even decrease MV concentrations during the recovery period (Wahl *et al.*, 2014). Conflicting results have also been observed after heavy intensity interval exercise (Walsh *et al.*, 2003; Guiraud *et al.*, 2013; Kirk *et al.*, 2013). The reasons for such inconsistencies are elusive, but may relate to differences in the exercise protocols, as well as pre and post-analytical procedures for MV quantification as no standard procedure currently exists. Hence, it seems that further experiments are required to conclusively determine the influence of exercise intensity on the appearance of circulating MVs.

2.3.4. *Putative exercise stimuli for microvesicle shedding*

The onset of rhythmic exercise brings about respiratory, haemodynamic, and neurohumoral responses to cope with greater cardiorespiratory, metabolic and thermoregulatory demands, so that enhancement of minute ventilation, cardiac output ($\dot{Q}$), and sympathetic outflow occurs in an intensity-dependent manner (Mortensen *et al.*, 2008; Laughlin *et al.*, 2012). As a consequence, exercise may promote the increase of several factors that have been shown to act as agonists of PMV and EMV release *in vitro* and may be hypothesised to be involved with the increased appearance of these MVs after exercise.

Cell culture experiments have provided insight regarding the impact of mechanical forces on platelet and endothelial cell MV formation, but studies in human models are still needed to integrate and better understand these responses in a complex physiological system. During exercise, the rise in metabolic demand requires a proportional oxygen supply to working muscles, which is attained through elevations in local blood perfusion and lead to a marked increase in vascular shear stress in the circulation of exercising limbs (Credeur *et al.*, 2010; Wray *et al.*, 2011). Augmented exercising limb perfusion accounts for the majority of the increase in $\dot{Q}$, but if exercise is prolonged an increase in shear stress may be observed even in arteries feeding non-active limbs (Tanaka *et al.*, 2006; Tinken *et al.*, 2010; Padilla *et al.*, 2011b), probably to facilitate heat dissipation from the skin. The systemic haemodynamic

response to exercise substantiates shear stress as a potential agonist for PMV formation, since *ex vivo* platelets have been shown to release MVs after shear stimulation (Miyazaki *et al.*, 1996; Reininger *et al.*, 2006).

In contrast to platelets, data from endothelial cell work suggest that low shear stress increases the release of EMVs (Vion *et al.*, 2013b), likely reflecting pathological vascular remodelling. That makes sense, since an imbalance between pathologic and physiologic shear stress has traditionally been considered one of the major elements implicated in atherosclerotic plaque formation, even though a recent review has questioned the robustness of the classical shear stress theory and its relation with atherosclerotic plaque development (Peiffer *et al.*, 2013). Current *in vivo* studies focusing on EMVs as biomarkers of vascular damage support this idea by demonstrating that experimentally-induced disturbed blood flow (BF) is coupled with increases in circulating [EMV] in healthy individuals (Jenkins *et al.*, 2013), and that vascular shear stress correlates negatively with the [EMV] in renal failure patients (Boulanger *et al.*, 2007). Hence, because shear stress increases with exercise, and EMV release has been reported to be inhibited through a NO-related pathway under high shear forces (Vion *et al.*, 2013b), it seems logical that increases in [EMV] observed in some studies after exercise is mediated by a shear-independent mechanism. Alternative, elevated transmural pressures during exercise could be a stimulatory mechanism for EMV release, since cultured pulmonary endothelial cells have been shown to shed MVs during progressively greater cyclic stretching (Vion *et al.*, 2013a). However, *in vivo* evidence is lacking.

Investigation of the effect of shear stress on MV formation with exercise is frequently restricted by intrinsic issues related the measurements of vascular shear forces during cycling or running due to movement of active limbs. In this context, exercise models engaging a limited muscle mass may be useful. The single leg knee extensor exercise engages a small amount of muscle mass (the quadriceps muscles) with the rest of the body remaining relatively stable. The experimental position enables easier access to lower limb major vessels in comparison to cycling or running (Andersen *et al.*, 1985), while eliciting significant local haemodynamic adjustments. For example, dynamic knee

extensor exercise can increase thigh perfusion to 7-8 $l\cdot min^{-1}$, a relatively high value compared to ~10 $l\cdot min^{-1}$ attained for the whole leg during maximal cycling (Mourtzakis *et al.*, 2004; Mortensen *et al.*, 2005; Calbet *et al.*, 2007; Mortensen *et al.*, 2008). Whether exercise engaging only a small muscle mass is a physiological stimulus capable of increasing MV concentration in the circulation is unknown.

A limitation with exercise models, however, relates to the increased metabolic demand and subsequent large humoral adjustments, which may be confounding factors when determining the sole influence of vascular shear stress on MV formation in humans. An alternative approach to this question could involve haemodynamic manipulations with slight metabolic influence through passive heat stress (Pearson *et al.*, 2011). Resting limb BF can be easily doubled with passive heating (Padilla *et al.*, 2011b; Pearson *et al.*, 2011). In addition, local BF manipulations are possible through regional limb heating and cooling (Chiesa *et al.*, 2015), with the benefits of small relative increases in circulating catecholamines (Kim *et al.*, 1979; Pearson *et al.*, 2011; Chiesa *et al.*, 2015; Gagnon *et al.*, 2015), which are known stimulants of PMV formation. One could speculate that the thermal stress could trigger platelet activation and MV release (Bouchama *et al.*, 2008), but evidence for *ex vivo* activation of platelets by temperature *per se* has not been supported between 37° C and 42° C (Gader *et al.*, 1990; Maurer-Spurej *et al.*, 2001), and seems to occur only when incubation temperature reaches values above those tolerable by conscious humans (43° C) (Gader *et al.*, 1990). This suggests that mild to moderate passive heat stress may serve as a method to explore the relevance of vascular shear stress in PMV formation in healthy humans.

Several biochemical factors, including cytokines, inflammatory agents and hormones, can also stimulate vesiculation in different cells. C-reactive protein (CRP) may increase after exercise sessions (Shojaei *et al.*, 2011), and it has been shown to induce EMV formation *in vitro* (Wang *et al.*, 2007b), as does thrombin (Sapet *et al.*, 2006), and angiotensin II (Burger *et al.*, 2011). Hepatic synthesis of CRP can occur through cytokine stimulation, mainly by interleukin (IL)-6 (Kasapis & Thompson, 2005). Hence, even if CRP has been found to act directly on endothelial cells by inducing EMV blebbing, it is conceivable that this

inflammatory protein may work in synergy with cytokines within a living organism.

Cytokines are involved in the acute response to exercise and IL-6 is the most responsive (Figure 2-5), displaying intensity and volume dependent increases in blood levels as exercise progresses (Scott *et al.*, 2011, 2013). Depletion of skeletal muscle glycogen stores is suggested to stimulate the expression of this cytokine in contracting muscles (Pedersen *et al.*, 2001; Steensberg *et al.*, 2001), which can reach the circulation and potentially increase glucose and free fatty acid release by the liver to support the greater substrate requirements of exercise (Pedersen *et al.*, 2001; Glund *et al.*, 2007). Besides its effects on the control of glucose homeostasis, IL-6 released during exercise is believed to modulate part of the anti-inflammatory/inflammatory balance to exercise by, for example, stimulating the delayed rise in circulating IL-1 receptor antagonist after the end of exercise (Scott *et al.*, 2011) and enhancing the hepatic release of CRP (Petersen & Pedersen, 2005). Moreover, IL-6 has been reported to directly stimulate PMV formation *ex vivo* (Nomura *et al.*, 2000), and might indirectly cause MV formation through the action of CRP observed in endothelial cells (Wang *et al.*, 2007b). The influence of exercise on circulating TNF-α concentrations is less clear (Kasapis & Thompson, 2005), but this cytokine has been used as a agonist to induce endothelial MV release in culture (Jimenez *et al.*, 2003). In any case, no information is currently available regarding whether the cytokine and/or inflammatory responses drive MV formation with exercise.
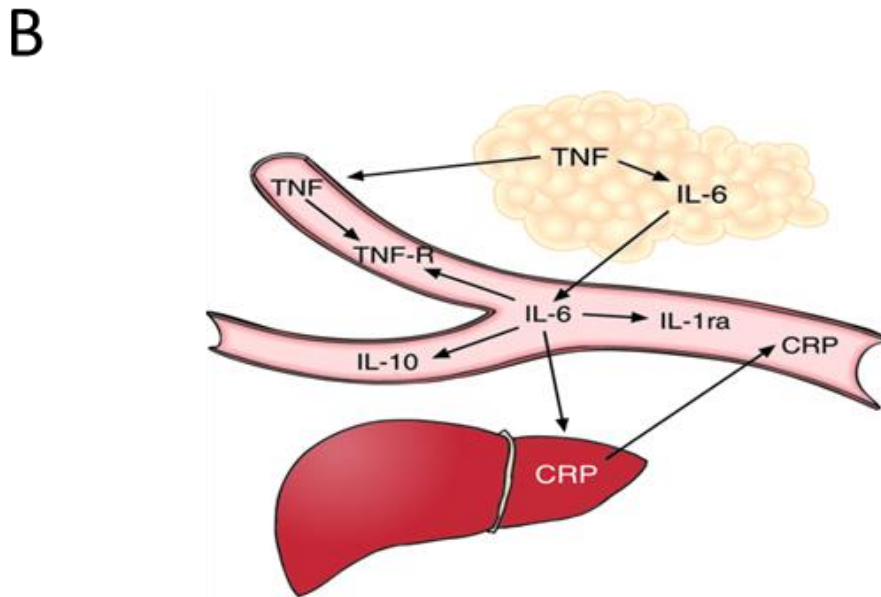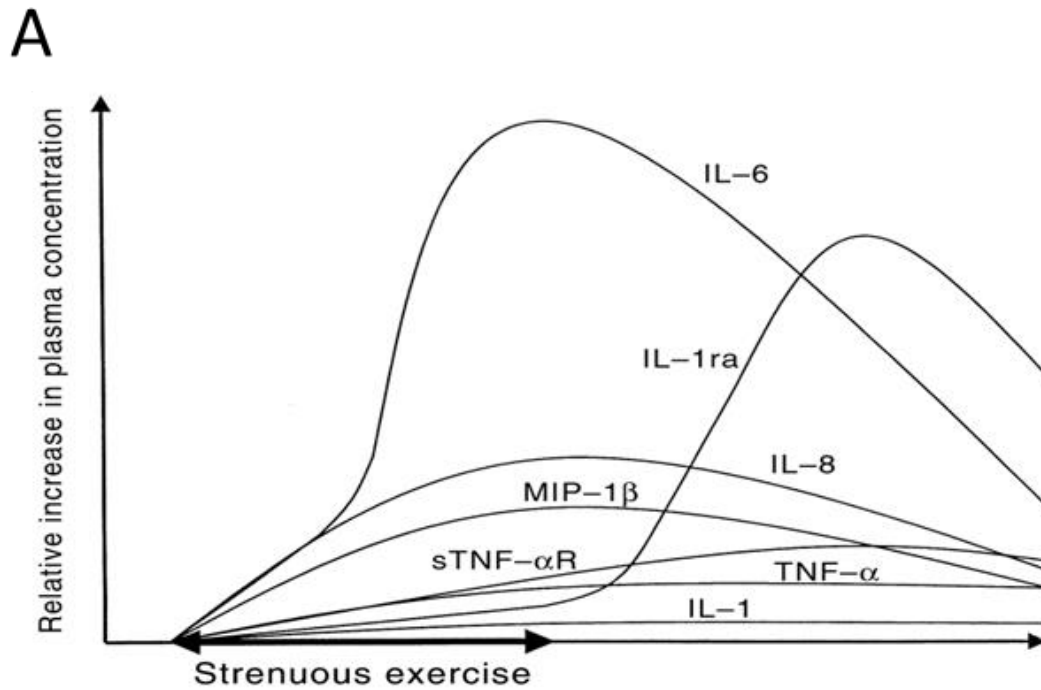
Figure 2-5. Schematic cytokine response to exercise (A), and subsequent influence on C-reactive protein (CRP) stimulation (B). IL interleukin; MIP-1β macrophage inflammatory protein 1β; TNF-α Tumour necrosis factor α; sTNF-αR soluble TNF-α receptor. Adapted from Pedersen (2000), and Petersen and Pedersen (2005).

Taken together, results from *in vitro* experiments indicate that several physiological haemodynamic and biochemical adjustments that are known to take place during exercise might trigger the shedding of PMVs and EMVs in the circulation of exercising humans (Figure 2-6). Whether an association between changes in MV concentrations and these putative agonists exists during exercise, however, still needs to be demonstrated.
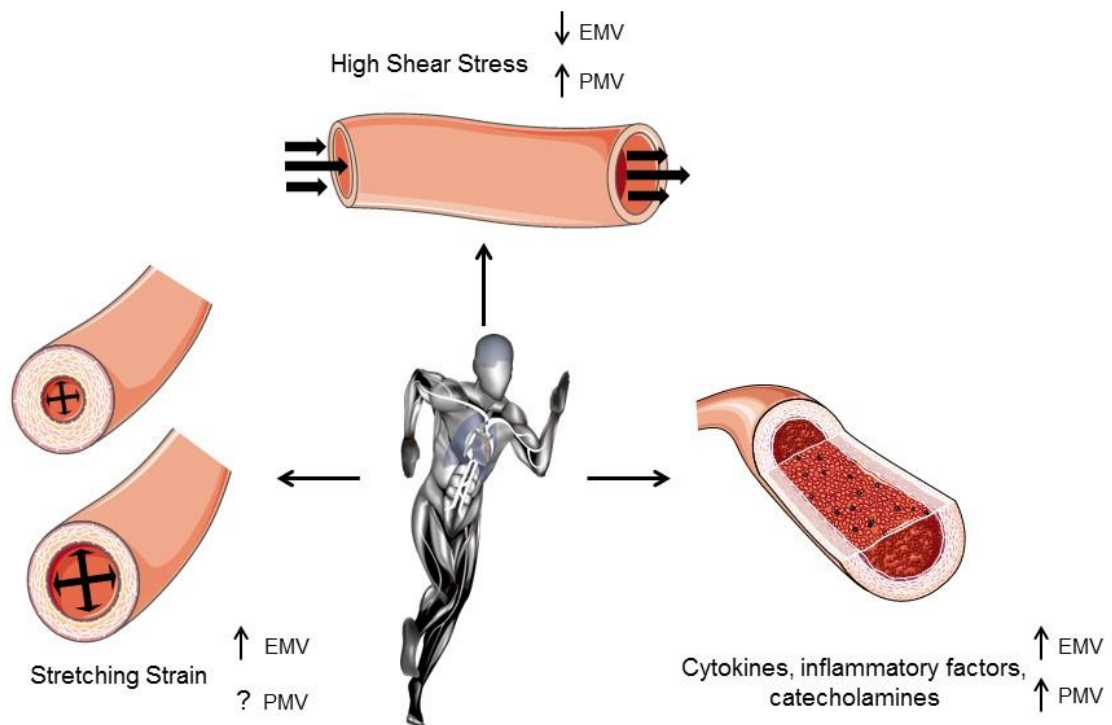
Figure 2-6. Hypothetical exercise-induced stimuli for platelet (PMV) and endothelial microvesicle (EMV) release. High vascular shear stress, certain cytokines and catecholamines are all putative stimuli for PMV production in response to exercise, whereas EMV formation may be stimulated by vascular cyclic stretching, as well as inflammatory cytokines and proteins (Miyazaki *et al.*, 1996; Nomura *et al.*, 2000; Jimenez *et al.*, 2003; Reininger *et al.*, 2006; Wang *et al.*, 2007b; Tschuor *et al.*, 2008; Vion *et al.*, 2013a; Vion *et al.*, 2013b). Figure produced using Servier Medical Art (www.servier.com).

2.4. Physiological function of exercise-derived microvesicles

Based on the above mentioned work, it seems logical that MVs produced during exercise would exhibit a variety of biological activities. However, very little information currently exists in this regard, with available research focusing exclusively on the potential involvement of MVs in haemostasis.

Platelets play a major role in haemostatic control, and the involvement of PMVs in this process has now been widely appreciated (Wolf, 1967; Connor *et al.*, 2010; Shantsila *et al.*, 2010). MVs may support coagulation by exposing haemostatic proteins in their outer membrane, such as tissue factor (Sossdorf *et al.*, 2011). Since PS may serve as a direct catalytic surface for thrombin formation, MVs harbouring PS on their outer leaflet can also support coagulation (Connor *et al.*, 2010), and an elevation in blood MV content would be expected to increase the blood thrombotic potential. Indeed, circulating MVs isolated after cycling have shown increased coagulation activity (Sossdorf *et al.*, 2010, 2011). One could assume that this was simply mediated by the greater concentration of circulating PMVs and other MVs post-exercise, but it worth noting that the MV kinetics and coagulant activity followed a different time-course, indicating that qualitative modifications in exercise-derived MVs may have also taken place. This finding agrees with the fact that thrombotic stimulation can occur during exercise (El-Sayed *et al.*, 2000; Yegutkin *et al.*, 2007; Maruyama *et al.*, 2012), but it is also important to recall that fibrinolysis may also be stimulated even during moderate efforts in healthy humans, thereby counteracting the risks of coagulation during exercise (El-Sayed *et al.*, 2000).

Therefore, the present literature provides evidence that MVs are involved in the complex haemostatic regulation in response to exercise. Further biological roles of these extracellular vesicles in the context of exercise are unknown, but since exercise training is a powerful physiological stimulus leading to a healthy vascular phenotype and that exercise may acutely increase the concentration of MVs in the circulation, it seems reasonable to theorise that exercise-produced MVs may distinguish themselves from those found with pathologic conditions and ultimately promote beneficial endothelial responses.

2.5. Conclusion

Cell-derived MVs have received growing attention in the scientific community due to their potential to serve as biomarkers of intracellular events and their innate biological activities. These extracellular vesicles had initially been thought to be a simple consequence of pathological disorders, and subsequently believed to play a role in maladaptation. More recent evidence has shown that MVs are not necessarily harmful, and are actually necessary for proper physiological function. Exercise seems to be a potent inductor of PMV, and possibly EMV, appearance in the circulation, but little information is available regarding their time-course of release and stimulus of formation particularly during exercise. However, *in vitro* and *in vivo* data combined suggest that vascular shear stress alongside certain biochemical agonists may trigger the release of MVs in response to exercise. Finally, a body of evidence indicates that these small particles may play a role in the complex haemostatic control with exercise, but the overall relevance of exercise-derived MVs requires further investigation.

Chapter 3 - Thesis Aims and Hypotheses

The general aims of this thesis were to investigate the dynamics of circulating PMVs and EMVs under different exercise stresses, to gain insights into putative mechanisms involved in their formation *in vivo*, and to explore the role of MVs produced during exercise as candidate mediators of endothelial responses *in vitro*. To do so, a sequence of human and cell culture-based experiments were performed as follow:

Study 1. Time-course of circulating microvesicle appearance during moderate and heavy intensity exercise and subsequent recovery

Study aims: Considering the need to characterise MV responses to traditional exercise sessions, the specific aim of study 1 was to describe the time-course of MV appearance in the circulation during and after heavy and moderate intensity prolonged cycling, since exercise intensity modulates the magnitude of change in putative agonists related to MV release. A secondary aim was to establish relationships between MV responses with haemodynamics and biochemical variables known to stimulate MV formation *in vitro* to gain insights into the role of these factors in the MV turnover during exercise and recovery.

Research hypotheses: In this study it would be expected to observe 1) an increase in plasma [PMV] during and after exercise, which is modulated by exercise intensity. 2) PMVs are expected to return to baseline in the late post-exercise recovery, and the concentration of PMVs during exercise are expected to correlate with changes in 3) vascular shear rate (SR), as well as plasma concentrations of 4) noradrenaline and 5) IL-6; whereas 6) circulating EMVs were expected to remain stable with submaximal exercise.

Study 2. Arteriovenous microvesicle dynamics with heat stress and exercise engaging a large or small muscle mass

Study aims: Platelets and endothelial cells have been shown to shed MVs under a variety of physiological stresses, including after exercise, but their arteriovenous dynamics and stimulus of formation with exercise are unknown. Hence, the aim of this study was to explore the dynamics of PMVs and EMVs in the arterial and venous circulation of exercising men, and to further examine whether changes in MVs would be closely related to alterations in vascular shear stress with passive heat stress, exercise and combined heat stress and exercise.

Research hypotheses: In relation of PMVs it is hypothesised that 1) circulating [PMV] would increase during whole-body passive heat stress and exercise; and that 2) localised increases in shear stress would coincide with local increases in venous PMVs during heating and exercise. On the other hand, 3) [EMV] would remain stable during passive heat stress but increase with very heavy large muscle mass exercise, when several endocrine factors are stimulated.

Study 3. Impact of circulating microvesicles produced during exercise upon endothelial cells: in vitro insights

Study aims: With exercise-derived MVs postulated in this thesis to play a positive role in endothelial cell adaptations, the aim of this study was to test whether incubation of HUVECs with MVs obtained during an exercise bout would stimulate migration, proliferation, wound-healing and morphological changes of endothelial cells.

Research hypothesis: Circulating MVs obtained during exercise would stimulate cultured human endothelial cells to 1) proliferate, 2) migrate, 3) heal from a scratch-wound, and 4) form structures related to angiogenesis *in vitro* at a greater extent than MVs obtained at rest.

Chapter 4 - General Methods

4.1. In vivo studies

4.1.1. Ethical approval

Ethical approval for each human study was obtained from Brunel University London Research Ethics Committee prior to the start of each study (Appendix I – Ethical Approval).

4.1.2. Participant recruitment

Healthy young men were recruited for *in vivo* experiments of this thesis. Participation in all studies was entirely voluntary and participants were informed about risks and procedures of the experiments, and were encouraged to ask questions regarding the respective studies. Verbal and written consent expressing interest in partaking in the research was obtained prior to the beginning of each study (Appendix II - Informed Consent) and a self-completed health questionnaire (Appendix III – Health Questionnaire) was completed by all participants. Volunteers with known cardiovascular, metabolic, and/or endocrine diseases were excluded.

4.1.3. Anthropometry

Anthropometric data were acquired prior to each study with participants wearing only light clothes, without shoes. Body mass was obtained with a digital scale (model 798, SECA, Germany) at 0.2 kg resolution and height was measured to the nearest 1 mm with a stadiometer (model 798, SECA, Germany). Body mass index was calculated by dividing body mass (in kg) by the squared height (in m) of each participant.

4.1.4. Maximal incremental test and spirometry

Maximal incremental tests were performed prior to main experimental visits using cycle ergometers or a custom-build knee extensor machine, according to the specific study. In general, after warming up in the test position participants performed a ramp or step incremental workrate test until volitional failure. Simultaneous breath-by-breath pulmonary gas exchange and ventilation were recorded using a metabolic cart (Quark B^2, Cosmed, Italy) in chapter 5 and chapter 6. The metabolic cart was calibrated before each test according to manufacturer's instructions, using certified gases of known O_2 (5%) and CO_2 (15%) concentrations. Oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were assessed by paramagnetic, and non-dispersive infrared gas analysers, respectively. Data analysis was performed on 10 second moving average curves and the maximum oxygen uptake ($\dot{V}O_2$max) was considered the highest value obtained during the incremental test. Concomitant HR measurements were recorded via a telemetric device (T31, Polar Electro, Finland). It is appreciated here that one can exercise at much higher workrate than that observed at $\dot{V}O_2$max, but for the sake of this thesis the term PPO will be used to refer to the power output attained at volitional fatigue during the maximal incremental test.

4.1.5. Principles of ultrasonography for cardiovascular measurements

Ultrasound devices are powerful machines used in clinical and research cardiovascular assessments and they work on the principle that transmitted ultrasound waves (usually between 2-15 MHz) can be reflected in human tissues as a consequence of change in ultrasound impedance between tissue boundaries. Returning echoes at specific frequency bands can then be captured and converted back to electric signal by a transducer (or probe) and displayed as 2D brightness mode (B-mode) ultrasound images. Typically, ultrasound probes are composed of piezoelectric crystals, which can transduce changes in voltage to generate mechanical waves (sound), and vice-versa. When transmitted waves travel through reflecting interfaces the returning echo signal

amplitude will depend on several variables, including the acoustic impedance difference between boundaries, ultrasound beam path angle, and tissue depth. For example, the greater the impedance difference, the bigger the echo reflection between tissues (Thrush & Hartshorne, 2010b).

The intensity of the returning echo can be reduced by a process known as attenuation, which increases as a function of depth and wave frequency. Due to the loss of signal energy because of ultrasound attenuation, high ultrasound frequencies have low penetration capacity and are used mostly for superficial tissue scanning. On the other hand, the axial resolution is increased as transmitted frequency increases, so the capacity to resolve between boundaries is improved (spatial resolution increases). As a consequence, most human superficial arteries can be investigated with ultrasound frequencies between 7.5-12MHz, whereas deeper structures such as abdominal organs can only be visualised using lower ultrasound frequencies but at the cost of spatial resolution.

The attenuation at different tissue depths can be corrected by fine adjustments of time gain compensation, which enables control of the amplification of the returning signal at specific time delays. Since it is known that deeper structures result in more attenuated signals and longer returning echoes, it is possible to increase the amplification of longer duration pulses so the displayed signal is compensated on the screen. This is possible because the generation of ultrasound image is based on received signal time delay and intensity. As the sound travels and reflects on a surface there will be a time delay until the echo reaches the transducer. This time between transmission and reception of the signal (t) depends on the velocity of the ultrasound (c) (generally assumed as 1540 m$\cdot$s^{-1} in soft tissues) enabling the calculation of the horizontal distance of a reflective structure as given:

$$Distance = \frac{t \cdot c}{2}$$

Hence, B-mode images can be reconstructed by expressing the returning signal intensities in grayscale on the screen based on their return times (Figure 4-1).
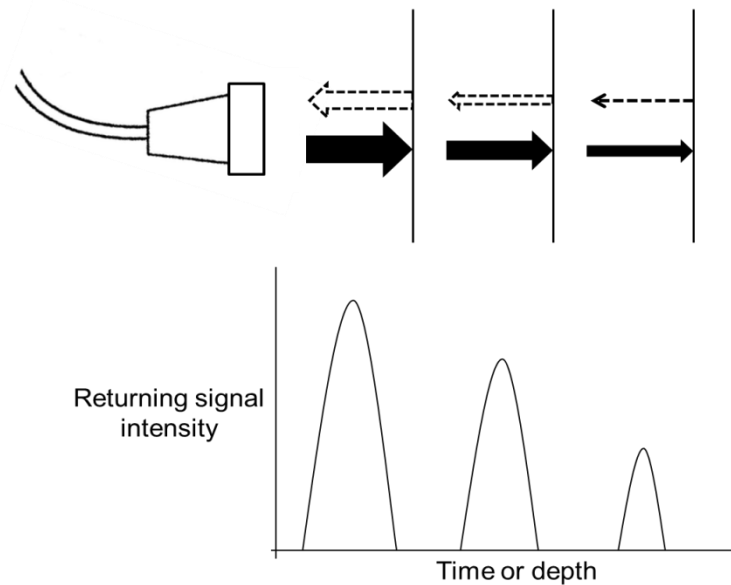
Figure 4-1. Example of probe transmitted wave and received specular reflection used to generate ultrasound images. Note that deeper structures result in longer returning times. The reflected energy is reduced due to signal attenuation. Adapted from Thrush and Hartshorne (2010b)

For blood velocity measurements the Doppler principle is applied. The Doppler effect was proposed by Christopher Doppler in middle of the 19th century and describes the change in sound wave frequency that occurs when the observer and/or the source of sound move towards or away from each other (Thrush & Hartshorne, 2010a). A typical example is usually given by an ambulance siren (source of sound) and a person (observer) outside the vehicle. If the observer remains stationary the sound wave length will reduce as the ambulance moves toward the person, increasing the wave frequency; whereas the wave length will increase (thus reducing sound wave frequency) as the source of sound moves away from the observer, explaining the change in audible sound when the siren passes by the observer. Conversely, movement of the observer can also influence the audible sound. In vascular

ultrasonography, the Doppler effect resulting from ultrasound interaction with clusters of moving erythrocytes enables the determination of the Doppler signal after demodulation and further analysis of its spectrum for quantification of blood velocity by the Doppler shift frequency, which is the difference between the transmitted frequency and the returning frequency and can be expressed as:

$$Doppler\ shift = \frac{2 \cdot V \cdot Ft \cdot Cos\theta}{c}$$

With V representing velocity of red blood cells; Ft the transmitted ultrasound frequency; Cosθ the angle of insonation; and c the velocity of ultrasound in the blood (typically assumed to be 1570 m·s^{-1}). The constant 2 refers to two Doppler effects, as first the transducer act as the source of sound and then as the observer of the returning waves reflected in the blood. As can be noted, changes in the insonation angle have important impact on the final result. Accordingly, if the wave beam is perpendicular to the blood (90°) the cosine is 0 and no velocity information can be obtained, whereas maximal information would be obtained if the probe were positioned parallel to the BF (cosine = 1). The latter condition is difficult to attain because ultrasound recordings are generally performed adjacent to the skin, limiting the ability to minimize the insonation angle due to anatomical limitations. Furthermore, if positioning the probe at 0° of BF direction were possible it would also result in no longitudinal B-mode imaging of the vessel, which is needed for several haemodynamic calculations. Thus an isonation angle of 60° or less is usually accepted as appropriate for blood Doppler measurements.

4.1.6. Limb haemodynamics

Measurements of limb BF and SR were made using a Doppler ultrasound device (Vivid 7, GE Logic, UK) according to the principles described

above, except during experiment 1 in chapter 6 where leg BF during cycling was obtained using the thermodilution technique (detailed in that specific chapter method section). Longitudinal duplex B-mode and pulsed-wave Doppler recordings were made at 22.3 frames per second and 8.5 MHz for B-mode; and 4.4 MHz for Doppler measurements, with a multi-frequency linear array transducer coated with water-soluble gel. The B-mode depth and probe position were adjusted to maximise image quality for each individual while keeping the Doppler insonation angle as low as possible (never greater than 60°). Doppler sample volume was adjusted to cover as much lumen as possible without extending beyond the vascular borders (Figure 4-2). Recordings were performed with participants seated upright or in a semi-recumbent position. Measurements at the brachial artery (BA) were made ~5-10 cm proximal to the antecubital fossa, whereas the common femoral artery, referred hereinafter as femoral artery (FA), was evaluated approximately 3 cm proximal to its bifurcation into deep profunda and superficial femoral arteries. Information from the FA was not recorded during cycling since the lower limb movement limits appropriate data acquisition.
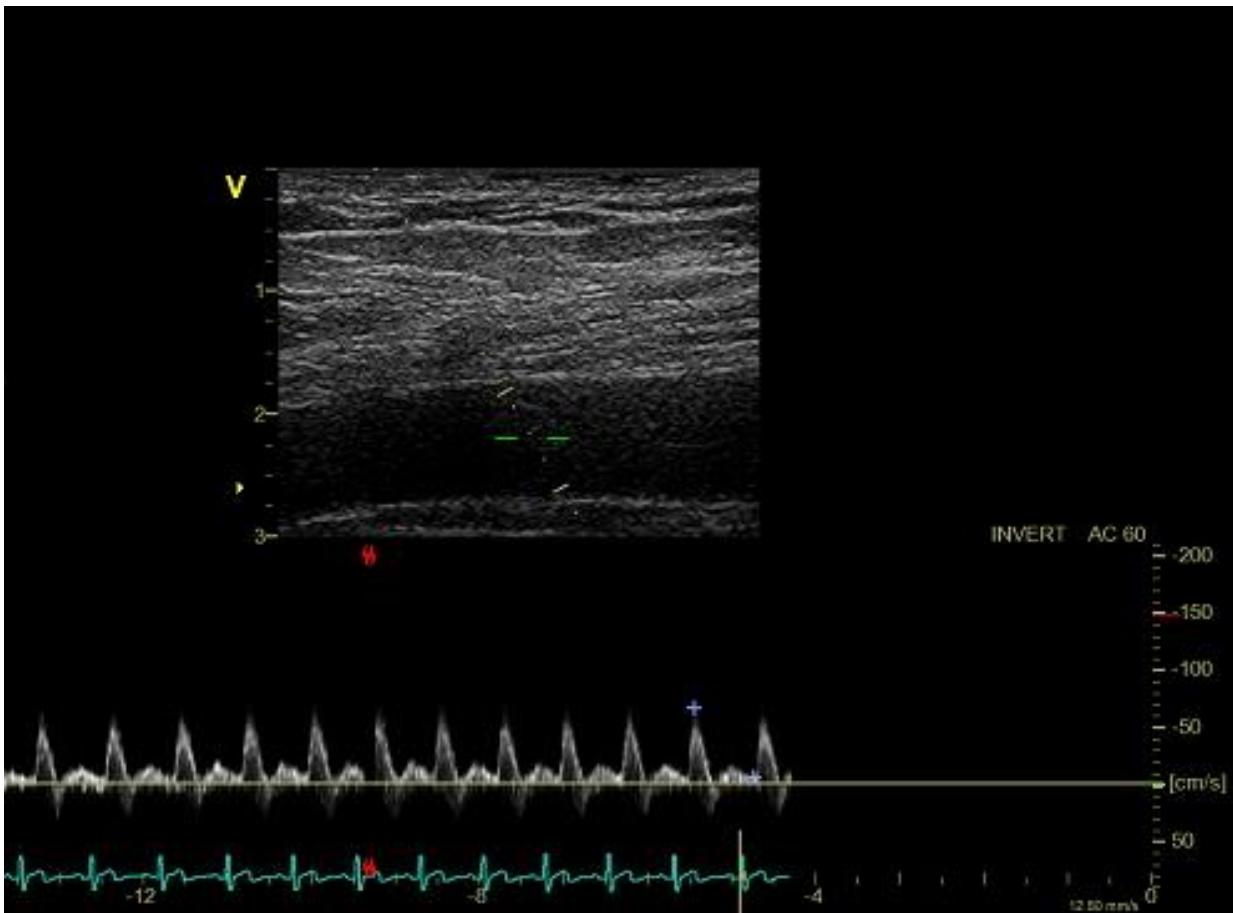
Figure 4-2. Example of duplex brightness mode and pulsed-wave Doppler from a common femoral artery. The arterial diameter and the mean blood velocity are derived from the ultrasound recording for calculation of blood flow and shear rate.

Off-line analyses were determined using specialised software (EchoPac, GE Logic, UK) on a personal computer workstation to obtain arterial diameters and Doppler blood velocities for further BF and SR calculations. Vessel diameter was traced manually from images acquired at end diastole at a site as close as possible to the Doppler sample volume. After visual inspection for quality spectral parameters, a 10 s weighted mean was obtained from the Doppler spectra for blood velocity measurement, with BF and SR calculated using the following equations (Credeur *et al.*, 2010; Jenkins *et al.*, 2013; Chiesa *et al.*, 2015):

$$Blood\ flow = Vmean \cdot \pi \cdot \left(\frac{D^2}{4}\right) \cdot 60$$

$$Shear\ rate = \frac{4 \cdot Vmean}{D}$$

Where Vmean is the weighted time-averaged mean Doppler velocity; and D is diastolic vessel diameter. At least 3 recordings were acquired at each time point with results averaged to obtain the final BF and SR values. Within-subject coefficient of variation of data obtained at rest in different days was 11% for BA BF, 6% for FA BF, 15% for BA SR, and 12% for FA BF (n = 9).

4.1.7. Cardiac output

In chapter 5, cardiac B-mode recordings using the same ultrasound device mentioned above were used for stroke volume (SV) and $\dot{Q}$ calculations. Apical four-chamber images were obtained with a phased array probe at 3.6 MHz and the frame rate was always greater than 60 frames per second, as adapted from echocardiographic recommendations (Lang *et al.*, 2006). To assist cardiac scanning, participants lay in the semi-decubitus position or were tilted during cardiac scanning periods when positioned on the semi-recumbent bike. Measurements were made between the 4th and 5th intercostal space along the mid-axillary line with the probe index marker rotated to the right (3 o'clock). A four-chamber view was assumed appropriate when all cardiac chambers were properly visualised alongside the septa, as well as the tricuspid and the mitral valves (Figure 4-3). When appropriate positioning and image quality were attained, the B-mode depth was adjusted to improve image quality and frame rate for data analysis. Complete cardiac cycles were recorded and participants were instructed about breathing manoeuvres to help data acquisition, which were most often required during exercise.
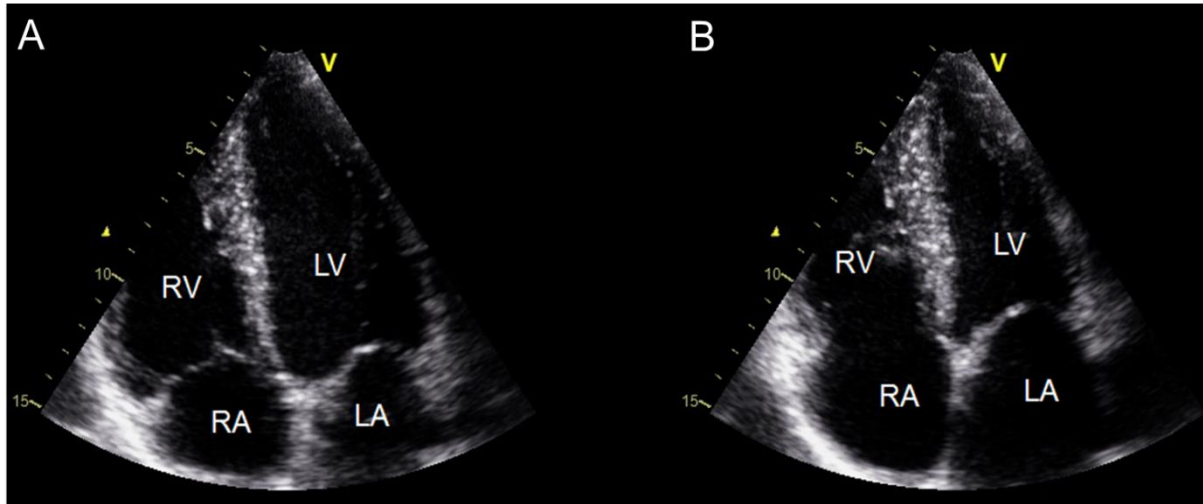
Figure 4-3. Apical four-chamber view at end diastole (A), and systole (B) used for stroke volume quantification. Due to probe positioning on apical plane and the basic acoustic wave reflection principle of echography, the images seem rotated rightwards. Hence the left ventricle (LV) is visualised on the right top of the image, and left atria (LA) on the bottom. The right ventricle (RV), and right atrium (RA) are shown in the left side.

Off-line analysis was performed again using the EchoPac PC station. The left ventricular endocardial cavity area was manually traced at end diastole and systole to enable ventricular volume calculation by the modified single plane Simpson's disc method. This method takes into account the geometrical changes in the ventricular shape, as a stack of discs of different sizes. The traced area is divided into disc sections of same height and known diameter, so the ventricular volume can be calculated as the sum of the disc volumes as described below:

$$Ventricular\ volume = \sum \frac{l}{n} \cdot \left[\pi \cdot \left(\frac{d}{2}\right)^2 \right]$$

Where l is base to apex length; n is number of discs; and d is disc diameter. The SV was obtained by subtracting ventricular end systolic volume (ESV) from end diastolic volume (EDV), whereas Q̇ was the product of SV

multiplied by HR. At least three cardiac cycles were analysed for each measurement. The between day within-subject coefficient of variation of $\dot{Q}$ obtained at rest was 5% (n = 6).

Participants' $\dot{Q}$ could not be obtained by echocardiographic measurements in chapter 6, but it was calculated using alternative approaches. In experiment 2, SV was acquired estimating aortic flow from direct intra-arterial radial pressure wave-forms applying the Modelflow method provided by BeatScope software (FMS, Netherlands), and HR was obtained by the analysis of the pressure-wave. In the first experiment, only baseline measurements could be obtained using the Modelflow method. Thus, $\dot{Q}$ during passive heat stress and exercise was calculated relying on Fick's equation using estimations of systemic O_2 extraction from directly measured limb O_2 extraction values (data not shown). This was based on the assumption that systemic and exercising limb O_2 extractions are strongly related (Mortensen *et al.*, 2005; Mortensen *et al.*, 2008). Hence, linear regression equations were used to estimate systemic O_2 extraction during heat stress and combined heat stress and exercise (A = 1.7322B - 76.126), and control exercise (A = 1.43B - 44.7), with variable A referring to systemic O_2 extraction and B denoting limb extraction. Estimated $\dot{Q}$ was subsequently calculated according using Fick's principle ($\dot{Q} = \dot{V}O_2$ / a-v O_2 dif; where a-v O_2 dif refers to arteriovenous difference for oxygen). Systemic $\dot{V}O_2$ was obtained using a metabolic cart (Quark B2, Cosmed, Italy).

4.1.8. *Catheterisation and blood sampling*

Blood sampling was carried out by arterial and/or venous access. Arterial catheterisation was performed by experienced clinicians in the BA or radial artery with the Seldinger technique, after local anaesthesia (2% lidocaine). Venous catheterisation was performed in an antecubital vein or in the common femoral vein. Samples were obtained without stasis in agreement with published recommendations for circulating MV acquisition. Before and after sample withdraw, the catheters were flushed with 0.9% sodium chloride solution (BD PosiFlush, Becton, Dickson and Company, USA) to maintain patency. The

first 3 ml of blood were discarded, and the collected blood was immediately mixed gently in tubes containing 0.129 mol·l^{-1} sodium citrate (Sigma-Aldrich, USA), and/or 8 mg ethylenediaminetetraacetic acid (EDTA) (Sarstedt, Germany).

Immediately after sample acquisition, platelet-rich plasma was prepared after whole blood centrifugation at 3,000 x g at 4°C for 10 min. Plasma from EDTA samples were aliquoted in tubes and then stored at -80°C for further analysis. Since platelet contamination can cause *de novo* formation of ex vivo MV, the supernatant from sodium citrate tubes was aliquoted into 1.5 ml vials and underwent a second centrifugation step at 15,000 x g and 4°C for 10 min to obtain platelet-poor plasma (PPP). The MV containing supernatant was removed carefully to avoid disturbing the platelet pellet and aliquoted in new vials for storage at -80°C until further experiments or analysis.

4.1.9. *Imaging flow cytometry overview*

Flow cytometric analysis of MV is one of the most common methods in the current literature and was the chosen technique for this thesis. Although predecessors of the modern flow cytometer had already been developed, the term "flow cytometry" was only introduced later during the mid-1970's (Zuba-Surma *et al.*, 2007). Flow cytometry (cytometry from the Greek roots *kutos:* vessel, container, or cell - in biology; and *metron:* to measure) refers to the measurement of cells under flow conditions (Shapiro, 2003), which can be done by combining a cell suspension with a laminar flow sheath stream. Cells are interrogated in the flow chamber by light sources, with the cells inducing forward scattering (FSC) and side scattering (SSC, or right angle scatter) of the light. The scattered light is detected by FSC and SSC detectors, and this provides information about the cells or particles within the sample. Fluorescence signals can also be detected after sequential low-high band pass filtration and signal decomposition, if fluorophore-stained samples are used.

Although powerful devices, traditional flow cytometers lack capacity for acquiring images of collected events, but innovative imaging flow cytometers

such as the ImageStream^X® Mark II (Amnis Corporation, USA) incorporate the remarkable feature of a fluorescence microscope within a flow cytometer (Figure 4-4), allowing visualisation at high throughput of individual events in the fluid path. This was made possible through intricate engineering that replaced photomultiplier detectors for charged-couple device cameras in the new imaging devices. This unique feature makes the ImageStream^X® a distinct data collection tool. Furthermore, the integrated IDEAS software (Amnis Corporation, USA) allows a myriad of analysis, ranging from typical object counts and population quantification by fluorescent signal intensity, to more sophisticated analysis such as the imaging of cellular protein co-localisation.
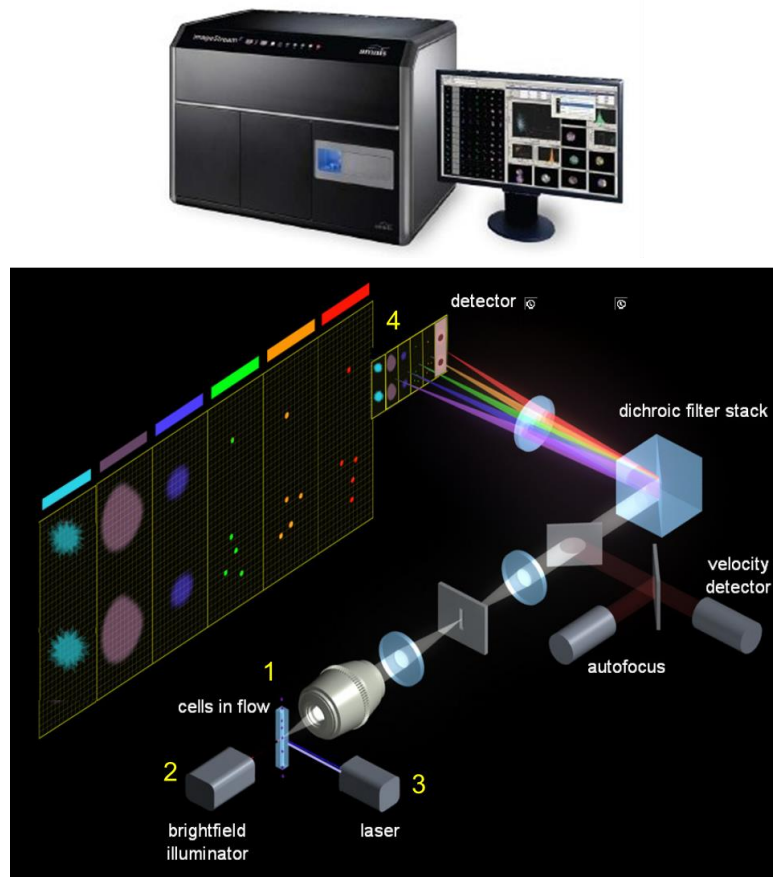
Figure 4-4. Overview of the ImageStream^X® Mark II flow cytometer system. The sample is hydrodynamically aligned in the flow chamber, running individually through the sheath path (1). Objects (*e.g.* microvesicles) are transilluminated as they cross the bright light illuminator generating forward scatter information (2), whereas side scatter, and fluorescent data are derived from a laser illuminator (3). The deflected light is decomposed into different spectral bands by the spectral decomposition element, reaching the charge-coupled device cameras at different angles, where the object information is recorded (4). Adapted from Amnis Corporation ImageStream^X® Guide, available at www.amnis.com.

The ability to discriminate between MVs of different origins currently relies on specific surface antigens to which fluorescent-conjugated antibodies can specifically bind. Antibodies are immunoglobulins produced by B cells in response to specific antigen-containing pathogens and help the immune system by various mechanisms, with the most outstanding characteristic of tagging foreign corpses for destruction. These Y-shaped proteins possess an antigen-binding site (or paratope) on top of their variable region on the heavy and light polypeptide chains (Figure 4-5, top). The selective antigen-antibody binding occurs when a specific region of the antigen known as an epitope interacts with the antibody paratope forming antigen-antibody complexes (Gleeson & Bosch, 2013). This specific binding capacity led to the development of commercially available antibodies (usually as immunoglobulin G class) to label cells and particles for biochemical analysis.

As mentioned in previous chapters, CD41 and CD62E are makers expressed by platelet and endothelial derived MVs, respectively. So fluorochrome conjugated anti-CD41 and anti-CD62E antibodies are common choices for the measurement of these MV subpopulations by flow cytometry. Each fluorochrome can be excited by light at defined wavelengths, absorbing energy and reacting by emitting light at a longer wavelength when electrons return to the ground state. This emission of light allows the quantification and discrimination between positive and negative events in flow cytometry.

An intrinsic issue associated with the use of fluorescent antibodies is non-specific binding. Even though the paratope and the epitope are supposed to selectively bind to each other, antibodies may also react non-specifically generating false-positive events. This process known as non-specific binding may happen for a variety of reasons, but a common cause is the interaction of the antibody fragment crystallisable (Fc) domain to the Fc receptor region of a molecule (Figure 4-5, bottom) (Hulspas *et al.*, 2009). Pre-incubating samples with Fc receptor blockers is thought to diminish this phenomenon, but one needs to bear in mind that a degree of non-specific binding will be present even after blocking techniques.
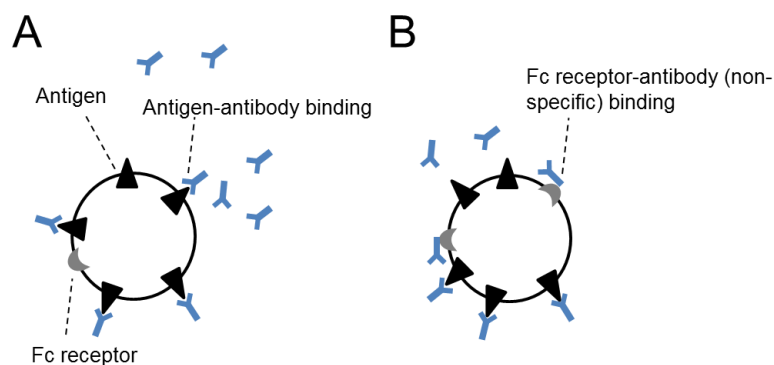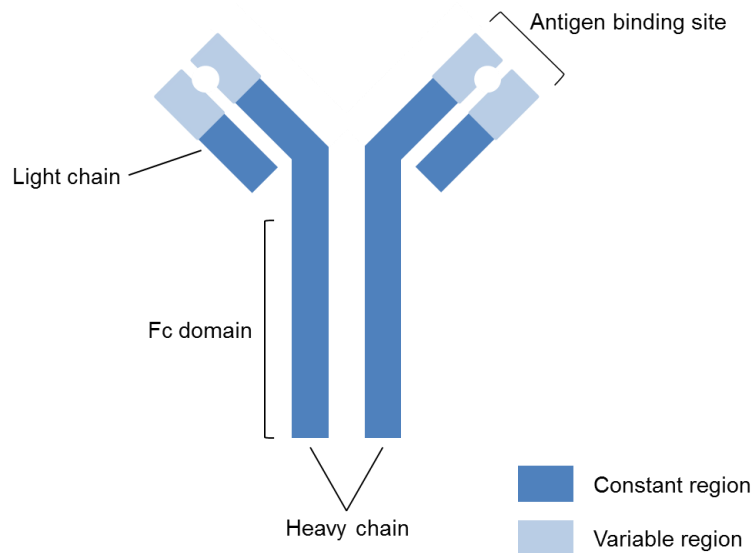
Figure 4-5. Schematic Y-shaped antibody structure, and antigen-antibody interactions. Top: The antigen binding site (paratope) is found in the variable region of the immunoglobulin, but the fragment crystallisable (Fc) domain may also interact with Fc receptors in the sample, resulting Fc-mediated antibody binding and false positive events. Bottom: The antibody paratope binds to the specific epitope region on the antigen allowing the identification of positive events if a fluorochrome conjugated antibody is stimulated at its specific wavelength (A). Non-specific interactions between the Fc receptor and the Fc domain in the antibody may increase the number of false positive events (B).

Although imaging flow cytometers are powerful devices for both photometric and morphometric analysis and present a much lower detection size limit compared to traditional cytometers, they also possess intrinsic limitations. For example, results from objects under analysis are derived from an automated mask placed upon the event. A mask defines the region from where specific feature information will be derived, and therefore inappropriate masking markedly impacts the features of the object under analysis, such as

diameter, area and aspect ratio (Figure 4-6). Misalignment of the object in the focal plane may also influence the results by generating blurred images that may overestimate the particle size, and intrinsic sample characteristics such as the objects contrast also can affect the acquired results. With this in mind, researchers must be aware of the device focal plane, sample flow velocity, and alignment during the data collection in order to minimise the acquisition of inappropriate events.
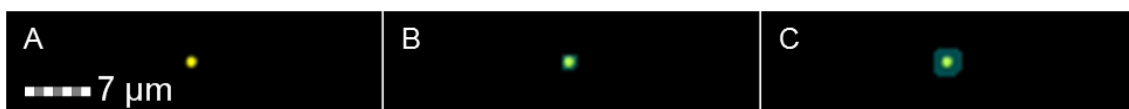
Figure 4-6. Example of a microvesicle event obtained through image flow cytometry and the influence of different masks on the determination of object features of a single microvesicle. A = no mask; B = Appropriate mask covering the entire object; C = inaccurate mask resulting in overestimated microvesicle features.

Due to the variety of techniques available for extracellular vesicle quantification, an important aspect in MV research is the agreement between measurement methods. van der Pol et al. (2014) examined the ability of five common methods for MV quantification, including traditional flow cytometry, to discriminate between particle diameter and concentration, using a known mixture of polystyrene calibration beads (from ~46 to 596 nm of diameter) and MV-rich biological fluids (urine). Compatible with the "iceberg theory" discussed in chapter 2, traditional cytometry underestimated unstained vesicle concentration by approximately 18-fold compared to dedicated submicrometer particle flow cytometry, and almost 300 fold in relation to the reference transmission electron microscopy, which resulted primarily from differences in the minimal detectable vesicle size capabilities of each technique (van der Pol *et al.*, 2014a). This demonstrates that agreement between absolute MV concentration values obtained using different methods is limited by the resolution capacity of the employed techniques. Hence, because the imagining flow cytometry technology used in the current thesis provides enhanced size detection capacity compared to standard flow cytometry (Headland *et al.*, 2014),

greater absolute MV concentrations were expected and observed in this thesis when compared to research using traditional flow cytometry.

4.1.10. Microvesicle quantification

On the day of analysis, PPP samples were thawed at room temperature, and 1.5 µl of PPP were pre-incubated for 10 min with 0.8 µl of TruStain Fc receptor blocker (BioLegend, USA). Next, samples were incubated in the dark for 25 min with PE conjugated anti-human CD62E (100 ng), and PE/Cy5 anti-human CD41 (10.2 ng) fluorescent antibodies (both from BioLegend, USA). Samples were then diluted in 195 µl of 0.2 µm filtered phosphate buffered saline (PBS) (Sigma-Aldrich, Germany) and centrifuged for 15 min at 17,960 x g and 4°C. This washing step was necessary to reduce the relatively high signal background arising from unbound CD62E. Following centrifugation, 170 µl of supernatant were carefully removed and the MV pellet was resuspended in an additional 30 µl PBS before analysis. Single-stained samples were analysed for each fluorochrome to set a compensation matrix for each sample.

The concentration of MVs was quantified using the ImageStream^X® Mark II flow cytometer (Amnis Corporation, USA) using the INSPIRE software (Amnis Corporation, USA), with 60 x magnification, 488 nm laser intensity at 100 mW, SSC laser intensity at 1.64 mW, using the lowest flow rate available to increase sensitivity while reducing the chance of doublet events. Laser intensities were determined through pilot experiments in order to maximize signal intensity but avoiding pixel saturation. Both FSC and SSC were acquired (on channels 1 and 6, respectively) and the fluorescent signal from each fluorochrome/marker was recorded in their specific wavelength channels. All events were collected, but sample acquisition was limited to no less than 1,000 events in PMV region. This gating strategy for data collection is a potent tool within the INSPIRE software, as it enhances the precision of the events of interest. The *a priori* choice for the PMV instead of EMV gate relied on previous evidence indicating that PMV are more responsive to an exercise stimulus than EMV.

Following acquisition, data were analysed off-line using IDEAS software (version 6.1, Amnis Corporation, USA). After single-staining compensation matrix correction of all files, an upper limit size threshold was delimited in a FSC by SSC dot plot based on 1 µm diameter calibration beads (Fluoresbrite, Polysciences, USA) to exclude non-MV events. Objects inside the size threshold were gated based on SSC intensity vs PE/Cy5 mean fluorescence intensity (for PMV), or PE mean fluorescence intensity (for EMV) (Figure 4-7). In the ImageStream^X® Mark II at 60 x magnification MVs present a very low distinct SSC, and form a linear cluster at low to moderate fluorescence intensities whereas flow calibration beads and other unlabelled events in the sample show a moderate to high SSC without fluorescence (Headland *et al.*, 2014). A threshold for positive events was set in each file based on the fluorescence minus one method (Hulspas *et al.*, 2009).
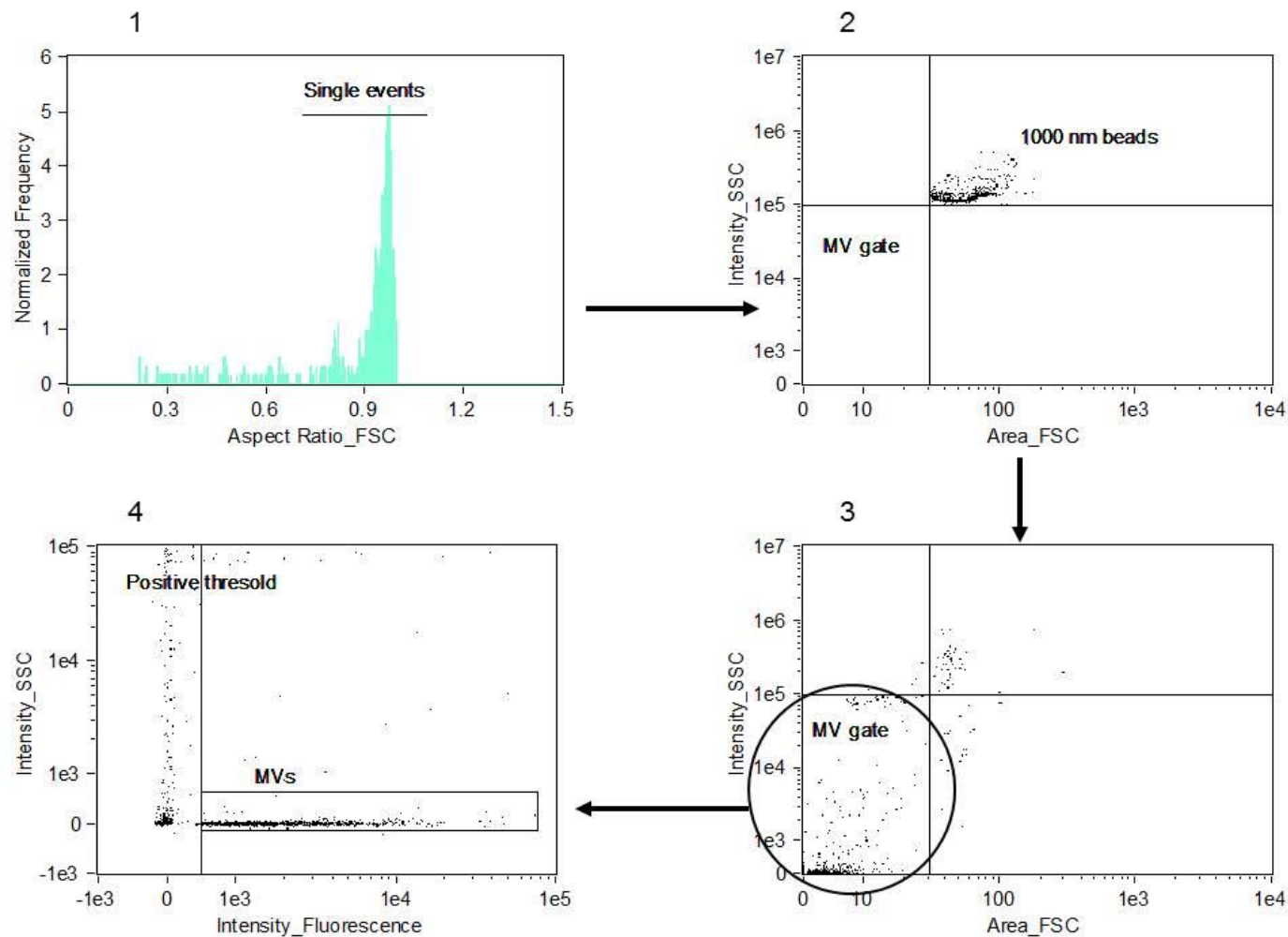
Figure 4-7. Illustration of gating strategy applied for image flow cytometric quantification of microvesicles (MVs). Calibration beads doublets presenting low mask aspect ratio are initially excluded (1), and 1 μm size calibration beads are used to determine a MV upper size gate (2) based on forward (FSC) and side scatter (SSC) features. This gate is applied in in platelet-poor plasma samples (3) to exclude large non-microvesicle events, and the quantification of MVs is performed based on SSC and fluorescence intensity dot-plots (4), where MVs agglomerate with small SSC but moderate fluorescence intensity (Headland *et al.*, 2014). Fluorescence minus one stained samples are used to set a fluorescence threshold for positive events, with those falling leftward the threshold representing ImageStream speed beads, and MVs negative for the antibody of relevance.

The ImageStream^X® Mark II quantifies the total sample volume used during analysis by way of a syringe pump used to inject the sample, hence the concentration of MVs was calculated by the software (in events·ml^{-1}) without the need for calibration beads as with other flow cytometer devices. Dilution factor corrections were performed after data acquisition to account for pre-analytical steps. Data obtained at rest in different days displayed a within-subject coefficient of variation of 15%, and 11% for PMVs and EMVs, respectively.

4.1.11.   *Enzyme-linked immunosorbent assay*

The ELISA was developed in the 1970's, and has become an important tool for immune system research and other fields of science. It is currently a standard method for the quantitative measurement of innumerable substances relevant to exercise physiology and sport sciences, such as hormones, cytokines, and even mRNA (Gleeson & Bosch, 2013).

Diverse types of ELISAs are commercially available, but the general principle of the assay relies on antigen-antibody binding interactions to immobilize pertinent molecules for further quantification. Concisely, antigens (or antibodies) are incubated with specific antibodies and enzyme-conjugated secondary antibodies (or antigens). An enzyme substrate is then added to the solution, which elicits a colour change by an enzymatic reaction and allows measurement of the relevant substance concentration by absorbance readings of light at specific wavelengths (Figure 4-8).
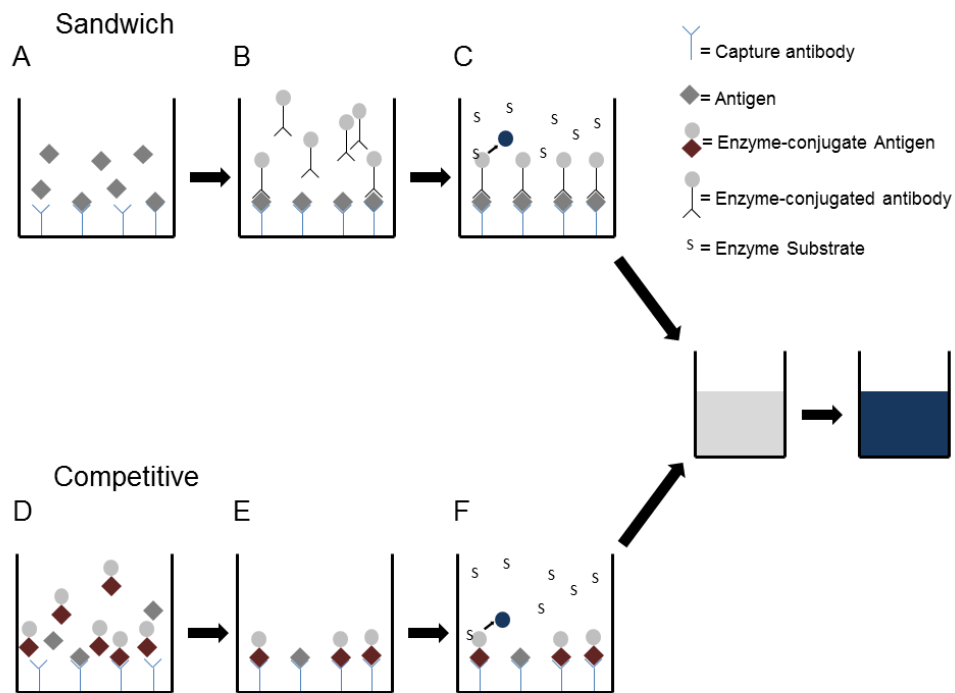
Figure 4-8. Sandwich and competitive enzyme-linked immunosorbent assay (ELISA) principles. In the sandwich ELISA the antigen of interest is added to a well-plate pre-coated with a specific capture antibodies (A), and then incubated with secondary enzyme-conjugated antibodies (B), followed by addition of the enzyme substrate solution (C) after appropriate washing steps. In competitive ELISA the antigens are incubated with enzyme-conjugated antigens (A) which compete for the specific capture antibodies (E) prior to adding the substrate solution (F). In either case the enzyme reacts with the substrate giving rise to colour change with resultant absorbance directly proportional to the antigen concentration in the sandwich ELISA, and inversely proportional to antigen concentration in the competitive version.

Sandwich ELISAs were used in the current thesis for soluble plasma molecule quantifications in chapter 5. Standards and samples were incubated in pre-coated microplates with immobilised mono-clonal antibodies against the relevant antigen, washed to remove unbound substances, incubated with specific enzyme-linked poly-clonal antibodies, and washed again. Next, the wells were incubated with the specific enzyme conjugates, followed by and substrate solution (and amplifier solution when relevant). Colour change reactions were stopped by addition an acid stop solution, and the light absorbance at relevant wavelength was determined using a plate reader (PB 800, Biohit, Finland). Final concentrations of each sample were calculated based on regression analysis from the known concentration standard *vs* optical density curve.

4.1.12.   *Plasma volume change*

Changing from a standing to the supine position, increasing hydrostatic pressure with exercise, and/or elevating sweating rates as a response to a rise in core temperature with exercise all cause plasma and blood volume to shift, which in turn affects the final concentration of plasmatic substances. As MV measured in the plasma were the main dependent variables in the human studies described in this thesis, correction for plasma shifts were performed in order to test whether increases in circulating MVs could be explained by reductions in plasma volume due to the interventions used in the thesis. Percentage of blood and plasma volume shifts were then calculated based on measurements of blood concentrations of haemoglobin (Hb) and the percentage of haematocrit (Hct) (Dill & Costill, 1974) as follow:

$$Blood\ Volume\ Change = \ 100 \times (Hb1 \div Hb2) - 100$$

$$Plasma\ Volume\ Change = \{(Hb1 \div Hb2) \times (100 - Hct2) \div [(100 - Hct1) - 1]\} \times 100$$

Where Hb1 and Hct1 stand for baseline Hb and Hct concentrations; with Hb2 and Hct2 referring to Hb and Hct of the next time-point of interest. Participants' Hct was measured using standard sodium-heparinized capillary tubes (micro-haematocrit tubes, Hawksley, UK) and centrifugation (HaematoSpin 1400, Hawksley, UK) procedures, and [Hb] was obtained through photometric analysis (HemoCue® Hb 201+ System, HemoCue AB, Sweden) in chapter 5; whereas Hct and blood concentration of Hb were determined using a blood gas analyser (ABL800, Radiometer, Denmark) of heparinized samples in chapter 6.

4.2. *In vitro studies*

All cell culture experiments were performed under sterile conditions, in a laminar-flow cabinet. Pipette tips were autoclaved prior to use, and local sterilisation was performed by swabbing gloves, flasks, laboratory equipment, and the cabinet with 70% ethanol. Basic biosafety procedures were followed according to the laboratory standards, and cells were incubated at 37°C and 5% CO_2, unless stated otherwise.

4.2.1. Cell culture medium

Growth medium for cell culture was prepared with M199 supplemented with 20% foetal bovine serum (FBS); 1% penicillin-streptomycin; 0.5% of 3 $g \cdot l^{-1}$ endothelial cell growth supplement; 2% of 1 $mol \cdot l^{-1}$ HEPES; 0.1% heparin; and 0.2% pyruvate. The culture medium was stored for up to four weeks at 4°C, and warmed up to 36°C in a water bath before being applied to cultures. Experimental medium was assay dependent and is described in chapter 7. A list of materials used for cell culture experiments is provided in the appendices (Appendix IV – List of Cell Culture Supplies).

4.2.2. Culture of human umbilical vein endothelial cells

Human endothelial cells are not easy to isolate because they are part of vessel walls and are not freely suspended in blood. A common method to investigate human endothelial responses *in vitro* is through the culture of HUVECs, which can be obtained for primary culture from human umbilical veins using collagenase. Once cultured, HUVECs attach to the substrate in a culture dish and change morphologically, forming a monolayer of polygonal shaped cells with a centrally located nucleus and present Weibel-Palade bodies and express von Willebrand factor (Hoyer *et al.*, 1973; Jaffe *et al.*, 1973b; Marin *et al.*, 2001). Due to the availability of this cell lineage, HUVEC culture was the selected method for the *in vitro* experiments presented in this thesis.

HUVECs were obtained as a generous donation from Harefield Hospital, North West London, and stored in liquid nitrogen until required for experiments.

For culturing, vials were quickly thawed in warm distilled water, and cells were gently transferred into culture flasks (T75, Sarsted, Germany), followed by slow dilution in growth medium to dilute the cryoprotectant whilst avoiding osmotic damage (Freshney, 2010b). Culture flasks were then incubated in a temperature (37°C) and CO_2 (5%) controlled incubation chamber, and all culture medium was changed 24 h after seeding the cells. Thereafter half medium changes were performed every 48-72 h.

Passaging was performed when monolayers became 60-80 % confluent using a 1:3 split ratio. To do so, the growth medium was removed and flasks were washed with 10 ml PBS. Then HUVECs were exposed to 1 ml of trypsin and incubated at 37°C for no longer than 2 min to detach adherent cells. Detachment was confirmed by microscopy and trypsin was deactivated with growth medium (supplemented with 20% FBS). Cells were transferred into 15 ml tubes and centrifuged for 6 min at 300 g and 18°C. Following centrifugation, the supernatant was discarded and the cell pellet was resuspended, seeded into new culture flasks in growth medium for sub-culturing, or used for experiments. Experiments were performed with HUVECs between passage 3-6.

4.2.3. Proliferation assay

Cell proliferation was measured using a modified tretazolium salt (MTT) assay. This colorimetric technique first developed by Tim Mosmann (1983) enables cell quantification as a consequence of the reduction of MTT to formazan crystals at an optical density of 570 nm. The MTT reduction to formazan is dependent on mitochondrial activity, and only occurs in living cells and increases in proportion to cell density (Mosmann, 1983) (Figure 4-9). A modified quicker MTT assay has been proposed in the presence of DMSO which solubilizes cells and absorbance readings can be acquired at 540 nm.

Cell densities were determined in a pilot experiment and the assay was performed by loading 50 µl of cell suspension (2,000 cells in MTT medium) into wells of gelatin pre-coated 96-well plates, followed by the addition of 50 µl of experimental medium. The plate was placed in the incubator for 48 h to allow

cells to proliferate. After incubation, 10 µl of MTT solution (12 mmol·l^{-1} diluted in PBS) was applied and gently mixed in each well. Cells were returned to the incubator for 4 h to allow formazan crystal formation, and then 80 µl of medium was removed from each well and replaced by 50 µl of DMSO to act as a solubilising agent. The plate was incubated again for an additional 10 min before absorbance readings were taken at 540 nm with a plate reader (ELx808, BioTek Instruments, USA). The average absorbance of quadruplicate wells was calculated for each condition, and results were expressed as a percentage of the negative control condition (*i.e.* MTT medium only).
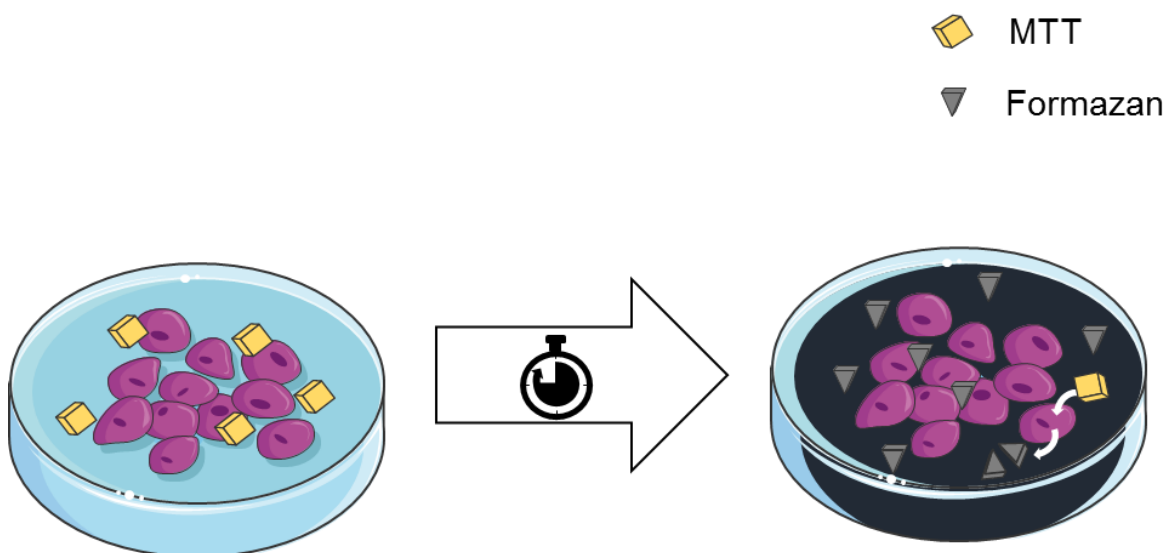
Figure 4-9. Representation of the proliferation MTT assay. Living cells convert tetrazolium salt (MTT) to formazan crystals, which increases light absorbance at specific wavelength. Thus, the greater cell density, the greater formazan production and respective light absorbance. Figure produced using Servier Medical Art (www.servier.com).

4.2.4. Cell migration assay

Cells migrate towards higher concentrations of chemical agents during a process called chemotaxis, and a number of assays were developed to test the chemoattractant potential of drugs or chemical agonists based on this kinetic principle. A typical chemotaxis assay, such as Boyden chamber-based experiments, involves applying a cell suspension onto culture inserts inside culture dishes loaded with potential chemoattractants. The insert is separated

from the dish by a membrane that allows cells to migrate towards the chemoattractant through its pores. In the current thesis a chemotaxis chamber (AP48 48 wells, Neuro Probe, USA) was used to test whether or not MVs may act as a chemoattractant agent for HUVECs. This protocol assessed the ability of serum starved cells loaded in an upper chamber to migrate through a polycarbonate filter (exposed filter area = 8 mm^2, pore diameter = 8 µm, pore density = 1,000 pores·mm^{-2}) toward chemoattractant-containing lower chamber (Figure 4-10).

On the day of the experiment, cells were serum starved in migration medium for 1 h, and pelleted by centrifugation after removal from the culture dish using trypsin. The wells of the lower chamber were loaded with 27 µl of experimental medium supplemented with MVs or control chemoattractants to form a slight meniscus and a 1% gelatin pre-coated polycarbonate filter was carefully positioned over the lower chamber. The upper chamber was firmly assembled and wells were loaded with 50 µl of FBS-free experimental medium containing HUVECs (25,000 cells). The chemotaxis chamber was incubated for 4 h at 37°C and 5% CO$_2$. Following incubation cells were removed from the non-migrated side of the filter by washing it in PBS and drawing the filter against a wiper blade 3 times. Migrated HUVECs were fixed in methanol for 10 min, and the filter was allowed to dry before it was stained in Giemsa stain (1:10 in distilled water) for 1 h. After staining, the filter was gently washed in distilled water, placed on a slide coverslip for analysis with an upright microscope (Axioskop 2, Zeiss, Germany) at 20x magnification. Migrated cells were counted manually using the software ImageJ (version 1.48, National Institutes of Health, USA), with the aid of a cell counter plugin. The average of triplicate wells was calculated for each condition, and the results were expressed as a percentage of the 10% FBS control condition, since no cells migrated in the negative control condition.
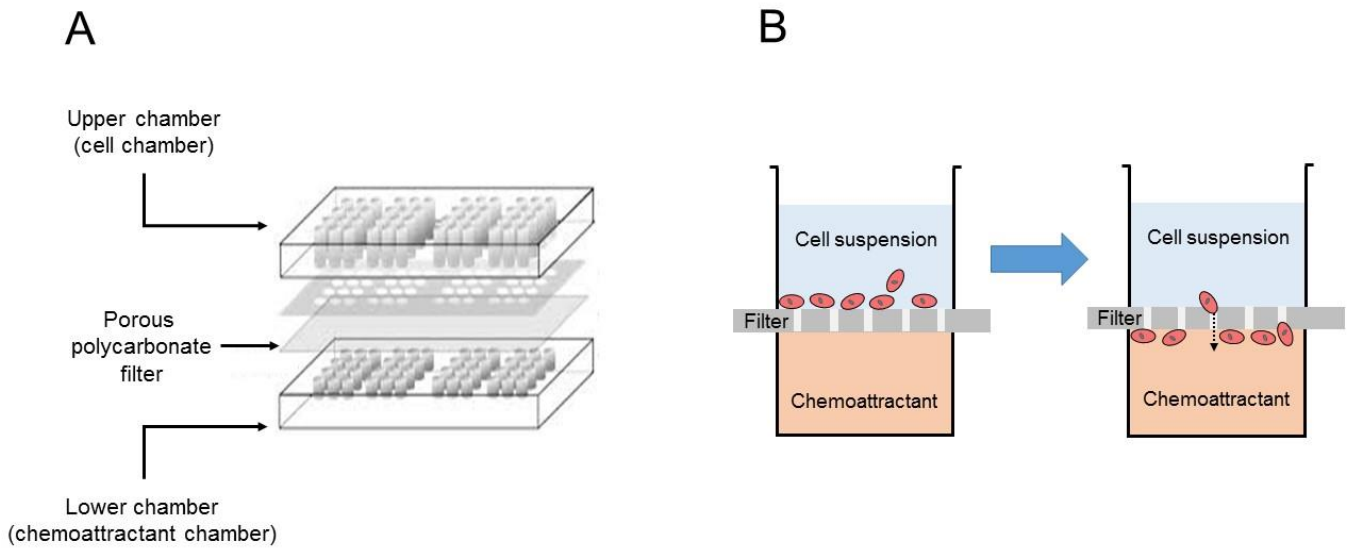
Figure 4-10. Diagram of the Neuro Probe chemotaxis chamber (A), and basic principle of the migration assay (B). Cells are loaded in the upper chamber and can migrate through the porous filter when chemoattractants are present in the lower chamber (B). Adapted from the Neuro Probe chamber manual.

4.2.5. Scratch wound-healing assay

Combined cell proliferative and migratory capacities can be investigated by tracking the closure of a cell free region by cell monolayers. Although a variety of assays are available (Valster *et al.*, 2005; Kramer *et al.*, 2013), a conventional and inexpensive method to track such responses is through the aptly described scratch wound-healing assay, in which a pipette tip is used to disrupt a continuous cellular monolayer by scratching the bottom of the well plate followed by imaging of the scratched area to track the wound closure (Liang *et al.*, 2007) (Figure 4-11).

Before the experiment a straight reference line was draw using a fine pen tip on the underside of each well of the 96-well plates. Wells were coated with 0.5% gelatin and incubated at 37°C. Cells were then cultivated in each well with 100 µl of growth medium until an almost confluent monolayer was apparent. On

the day of the experiment, HUVECs were serum starved by gently washing the wells with PBS and incubating the plate for 4 h with FBS-free medium. After starvation, sterile pipette tips (1-200 µl) were used to create a straight scratch of the cell monolayer, and all medium was replaced by experimental medium. The cell free area was imaged at 10x magnification at baseline and after 3.5 h of incubation, using an inverted microscope (Axiovert 200M, Zeiss, Germany). The incubation period was selected based on pilot experiments, to ensure that the wound would not be completely closed by the second time-point (which was found to occur in some conditions between 4 and 6 h).

The wound closure was measured using the software ImageJ (version 1.48, National Institutes of Health, USA). The cell free area was measured using polygonal selections, taking into account the reference line in the bottom of the wells. The closed area was calculated as the baseline cell free area minus the final cell free area measured after incubation. The average of quadruplicate wells was calculated and this was expressed as a percentage of wound closure relative to the negative control condition.
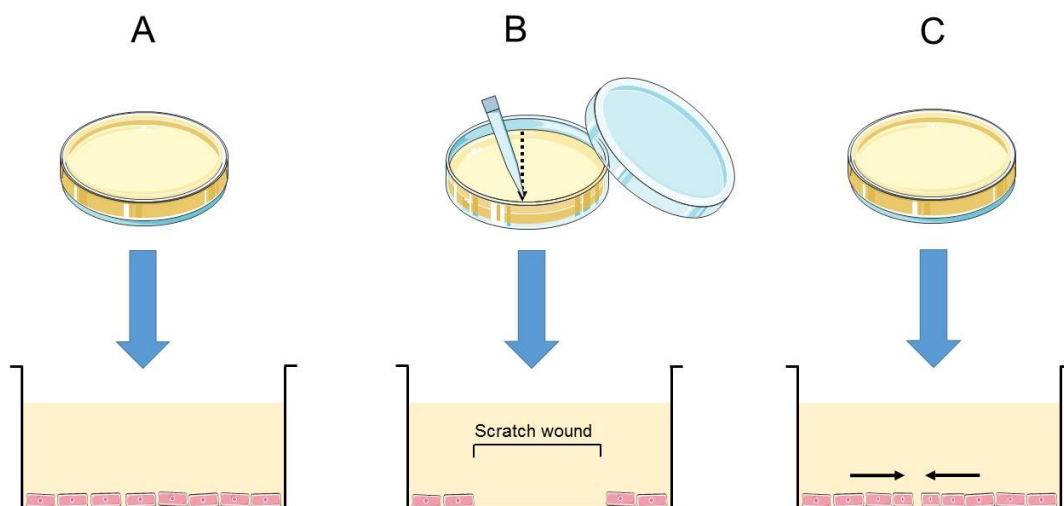
Figure 4-11. Illustration of the scratch wound-healing assay. A cell monolayer is cultivated until become almost confluent (A). The cell monolayer is then disturbed through a scratch wound using a pipet tip (B). Cells migrate and proliferate towards the cell-free region, analogue to a wound-healing process (C). Figure produced using Servier Medical Art (www.servier.com).

4.2.6. Tubule formation assay

In the presence of angiogenic factors, endothelial cells seeded on basement membrane proteins disrupt the membrane and migrate towards various chemoattractants, where they proliferate and undertake cytoskeletal reorganisation to form new vessels. A well-established, simplified *in vitro* model to study the angiogenic potential of biochemical candidates is the tubule formation assay. When specific extracellular matrix proteins (commercially available as Matrigel® or Geltrex® for example) are used as a substrate, seeded endothelial cells may migrate, proliferate, and change morphologically forming tubule-like structures in the presence of angiogenic agents (Figure 4-12). In order to investigate the putative angiogenic potential of exercise-derived MVs, an adaptation of the angiogenesis assay using a thin layer substrate (Faulkner *et al.*, 2014) was performed to quantify the formation of tubule-like structures.

In these experiments, 96-well plates were kept on ice and loaded with 78 µl per cm^2 of Geltrex® (Life Technologies, UK). To ensure even coating in each well, Geltrex® was spread using a sterile Eppendorf 0.5 ml combitip insert. The plate was placed into the incubator at 37°C for 30 min. Subsequently, serum starved cells (1 h) were suspended in experimental medium (M199 with penicillin-streptomycin and 0.5% FBS) and 50 µl were loaded into each well (10,000 cells per well). Then, 50 µl of conditioned medium (see specific methods section) were added to each well and the plate was incubated for 24 h. Wells were imaged after incubation using the Axiovert microscope at 5x magnification. The number of tubule-like structures and branching points of 3 fields of view per well were quantified using ImageJ software (version 1.48, National Institutes of Health, USA). Results were averaged from triplicate wells, and expressed as a percentage of the negative control condition.
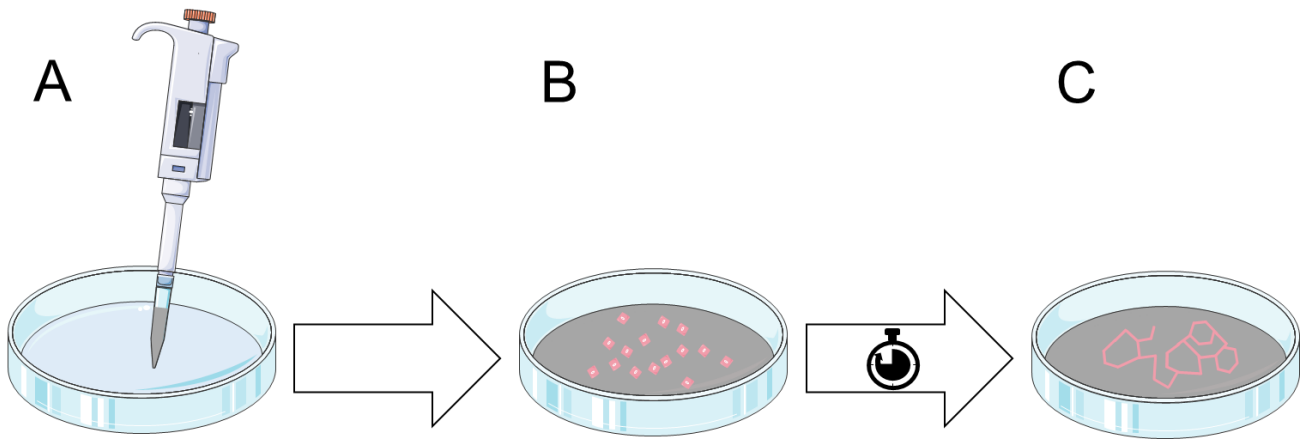
Figure 4-12. Example of tubule formation assay. An extracellular membrane protein solution is loaded into a culture plate (A), and endothelial cells are seeded onto the solidified membrane (B). In the presence of pro-angiogenic factors endothelial cells will change structurally to form tubule-like structures (C). The greater the tubule formation, the greater *in vitro* angiogenic potential of a drug or substance. Figure produced using Servier Medical Art (www.servier.com).

Chapter 5 - Study 1: Time-course of Circulating Microvesicle Appearance during Moderate and Heavy Intensity Exercise and Subsequent Recovery

5.1. Abstract

Exercise-induced increases in circulating PMVs have been observed after strenuous bouts of exercise, with less consistent results regarding EMVs. The dynamics of MV appearance and its causative mechanisms, however, are currently unknown. To characterise such phenomena, 9 physically active, young, healthy men (25 ± 4 years, mean ± standard deviation (SD)) had haemodynamic measurements and blood samples taken during a 4 h rest control trial, a moderate intensity exercise trial, and a heavy intensity exercise trial. Exercise visits comprised 1 h of cycling followed by 3 h of recovery. Plasma [PMV] and [EMV] were determined by imaging flow cytometry, whilst noradrenaline and interleukin-6 concentrations were measured by enzyme-linked immunosorbent assay. Mean vascular SR was acquired at the brachial artery by duplex Doppler ultrasonography. Heavy cycling caused a ~2-fold increase in plasma PMVs, which remained elevated at 1 h of recovery ($P <$ 0.05), but no changes were found in EMVs ($P ≥$ 0.05). Circulating MV concentrations remained stable throughout the control visit. [PMV] at baseline and during exercise were correlated to changes in arm SR during exercise ($P <$ 0.05; R^2 = 0.43), and to a lesser extent with plasma noradrenaline concentrations ($P <$ 0.05; R^2 = 0.21). In conclusion, circulating PMVs are likely to be increased in healthy humans during heavy intensity submaximal exercise, whereas [PMV] and [EMV] are unchanged during rest control. Moreover, this study demonstrates positive correlations between plasma PMVs, haemodynamics variables, and plasma noradrenaline concentrations during exercise, suggesting that elevations in vascular shear stress and catecholamines may be potential mechanisms underlying the exercise-induced plasma PMV appearance.

5.2. Introduction

Exercise is a physiological stress capable of stimulating vesiculation in a variety of cells, as observed by increases in circulating MV concentrations after running and cycling bouts (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Maruyama *et al.*, 2012; Kirk *et al.*, 2013). MVs derived from platelets seem to be the most responsive subpopulation, with studies consistently reporting a rise in venous PMVs after exercise bouts (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Maruyama *et al.*, 2012), but elevations in EMVs (Sossdorf *et al.*, 2011; Kirk *et al.*, 2013; Lansford *et al.*, 2015) and those derived from monocytes (Sossdorf *et al.*, 2011) have only been observed under certain conditions. However, exercise variables that modulate the release of MVs in response to exercise remain unknown.

Several haemodynamic and endocrine adjustments are influenced by the intensity of exercise. For example as exercise workload increases, greater perfusion is observed in the active-muscles (Rådegran, 1997; Saltin *et al.*, 1998; Calbet *et al.*, 2007; Mortensen *et al.*, 2008; Chiesa *et al.*, 2015), which occurs mostly through a rise in blood velocity at arterial level (Rådegran, 1997) and leads to proportional increases in arterial vascular shear stress. Likewise, sympathoadrenergic activity has been classically linked to exercise intensity (Galbo *et al.*, 1975), and the inflammatory response to exercise also seems to be dependent on intensity, as plasma cytokine levels tend to rise with relative workrate (Scott *et al.*, 2011). Increases in shear stress, noradrenaline, and IL-6 concentrations have all been shown to stimulate platelet vesiculation *in vitro* (Miyazaki *et al.*, 1996; Nomura *et al.*, 2000; Reininger *et al.*, 2006; Tschuor *et al.*, 2008), whereas endothelial cells seem to increase MV release under low shear conditions and in response to inflammatory cytokines (Jimenez *et al.*, 2003; Wang *et al.*, 2007b; Vion *et al.*, 2013b). Hence, relative exercise intensity may be an important exercise parameter influencing MV formation.

The time-course of blood MV appearance in response to exercise has been overlooked. Studies investigating circulating PMVs have only explored their appearance during post exercise recovery (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Maruyama *et al.*, 2012). Taken together, the

results from these studies indicate that PMVs may reach peak values within the initial hour of recovery, but it is possible that an increase in the appearance of these MVs may become evident during exercise. The EMV responses to exercise are less clear, with inconsistent results reported in the literature (Mobius-Winkler *et al.*, 2009; Sossdorf *et al.*, 2010, 2011; Guiraud *et al.*, 2013; Kirk *et al.*, 2013; Ross *et al.*, 2014; Wahl *et al.*, 2014; Lansford *et al.*, 2015), but submaximal exercise would be expected to induce smaller, if any, effect on this MV population.

The aim of this study, therefore, was to characterise the time-course of MV appearance during and after typical prolonged endurance exercise protocols. To do so, participants performed 1 h of moderate intensity and heavy intensity cycling bouts followed by 3 h of recovery with measurements throughout the experimental protocol on separate occasions. The *a priori* hypotheses were that [PMV], but not [EMV], would increase progressively during exercise and early recovery, according to exercise intensity; and that PMV changes during exercise would be related to haemodynamic (shear stress) and biochemical (*e.g.* noradrenaline and cytokines) mediators.

5.3. Methods

5.3.1. Participants

Nine young healthy males completed all five visits for this study. Participant characteristics are shown in Table 5-1. Volunteers were young lean men, mostly involved in recreational physical activities (self-reported).

Table 5-1. Study 1 participants' characteristics

Age (years)	25±4
Height (cm)	179±9
Body mass (kg)	80±12
BMI (kg·m^{-2})	25±2
$\dot{V}O_2$max (l·min^{-1})	3.3±0.7
HRmax (beats·min^{-1})	180±9
VT1 (l·min^{-1})	2.0±0.3
PPO (W)	259±37
MI workrate (W)	94±18
HI workrate (W)	160±25

Mean±SD for 9 participants; BMI, body mass index; $\dot{V}O_2$max, maximum oxygen uptake; HRmax, maximal heart rate; VT1, oxygen uptake at first ventilatory threshold; PPO, peak power output; MI, moderate intensity; HI, heavy intensity.

5.3.2. Experimental design

After recruitment, all participants eligible for the study were familiarised with the cycle ergometer and additional experimental procedures. During this session, participants performed a maximal incremental exercise protocol to minimise possible learning effects at the subsequent maximal test visit. After at least 48 h of recovery following the familiarisation visit participants returned to the laboratory to have their $\dot{V}O_2$max and derived variables determined using a ramp incremental test on a semi-recumbent cycle ergometer (Angio, Lode, Netherlands, Figure 5-1) with continuous collection of expired gases using a metabolic cart (Quark B2, Cosmed, Italy). Briefly, the test started with participants cycling at 80 revolutions per min (rpm) at a power output of 25 W with a ramp slope of 25 W·min^{-1}. They cycled continuously until the limit of exercise tolerance (i.e. the point when participants could not maintain a cadence ≥ 60 rpm for 3 continuous seconds). Participants then rested for 5 min and underwent a confirmatory step test to exhaustion at 5% above participants' ramp PPO. This step protocol served as a validation procedure to confirm that

participants reached their $\dot{V}O_2max$ (Rossiter *et al.*, 2006). Data were analysed using 10 s moving average window and the $\dot{V}O_2max$ was determined as the highest $\dot{V}O_2$ obtained during the incremental test. For VT1 determination a two criteria determination method was used, comprising the first deflection point in the $\dot{V}O_2$-$\dot{V}CO_2$ curve, and the point when the ventilatory equivalent of $\dot{V}O_2$ increased without concomitant rise in the respective $\dot{V}CO_2$ equivalent (Caiozzo *et al.*, 1982).

Figure 5-1. Semi-recumbent ergometer used for the moderate and heavy intensity exercise trials. The Angio unit can be tilted for echocardiographic measurements during exercise

The main experimental visits are summarised in Figure 5-2. Participants arrived at the laboratory in the morning, approximately 1.5 h after a light breakfast and rested for approximately 30 min while they were instrumented. The first measurement (0 min at Figure 5-2) took place around 20 min after cannulation. During the control day (CON) participants rested in a semi-recumbent position throughout a 240 min period while blood samples and additional measurements were obtained. The two last days were the exercise visits, which were performed in a randomised order. At the moderate intensity exercise trial (MI), 60 min of cycling at 80% of the workrate relative to VT1 was performed followed by 180 min of rest (recovery period), whereas during the heavy exercise intensity trial (HI) the participants cycled at VT1 power plus 30% of the difference between the workrate relative to their individual VT1 and $\dot{V}O_2max$ (*i.e.* delta concept). The delta method was selected for the heavy

intensity protocol because it has been shown to reduce the variability of physiological response between subjects (Lansley et al., 2011), and the classification of intensity domains as moderate and heavy exercise was based on previous definitions (Whipp & Rossiter, 2005). The three main experimental visits were separated by at least one week to reduce potential effects of blood drawing.
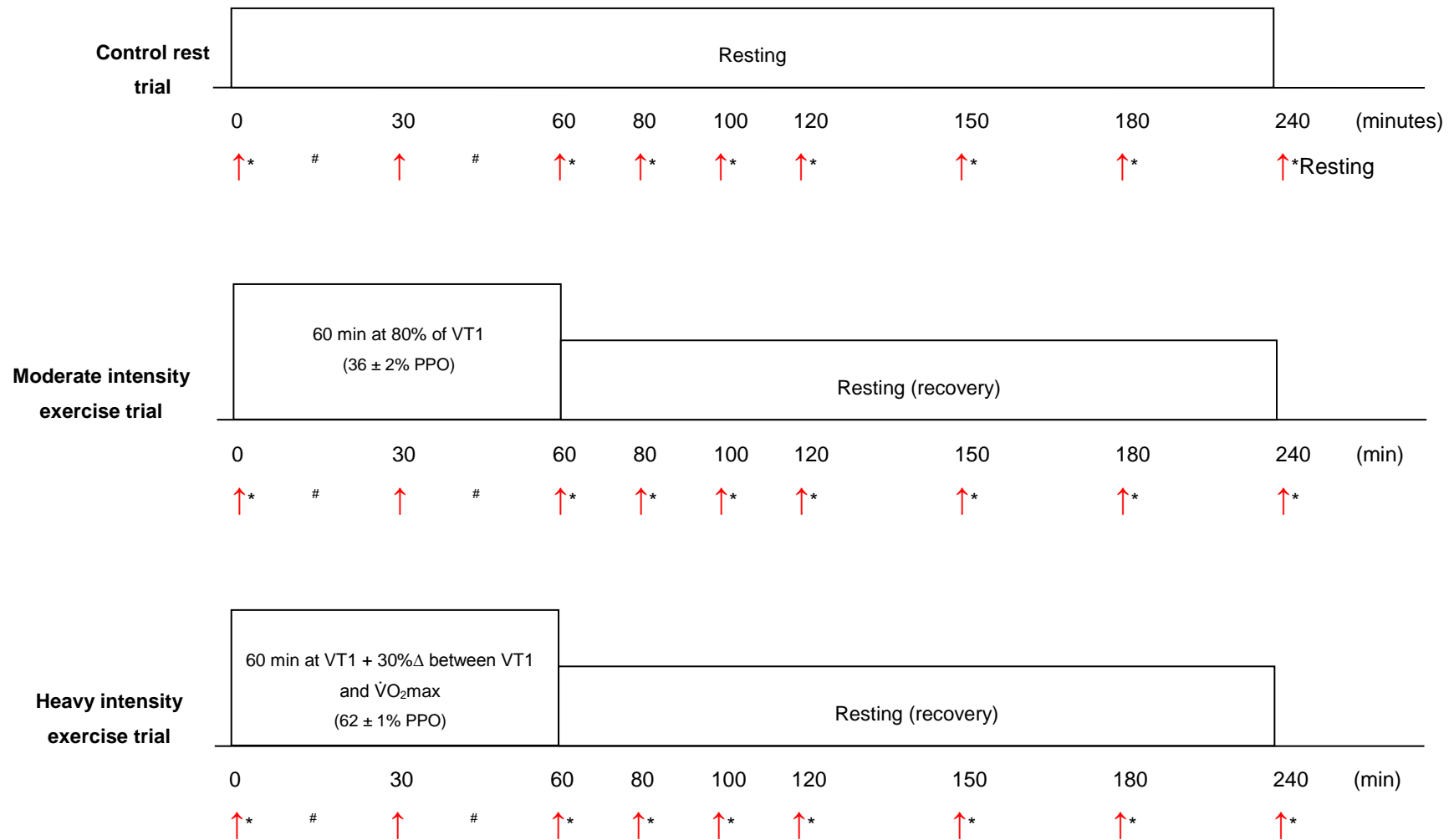
Figure 5-2: Time frame of data collection in the main experimental trials of study 1. Control trial is depicted on top, whereas exercise trials are in the bottom. ↑ blood sample; * cardiac output, femoral and brachial artery blood flow; # cardiac output, and brachial artery blood flow; VT1, first ventilatory threshold; $\dot{V}O_2$max, maximal oxygen uptake; PPO, peak power output.

5.3.3. Catheter placement and blood sampling

Venous blood samples were taken at 9 time points to determine the time-course of MV appearance in plasma at rest (Figure 5-2, top), as well as during and after moderate or heavy intensity exercise (Figure 5-2, middle and bottom). After arrival, participants rested in the supine position in a quiet room and an 18-gauge cannula (BD Venflon, Becton, Dickson and Company, USA) was inserted into a superficial antecubital vein of the arm by a trained researcher. Samples were obtained without venous stasis and the first 3 ml of blood were discarded. Approximately 15 ml of blood was aliquoted and stored at each time point. A 0.9% sodium chloride solution (BD PosiFlush, Becton, Dickson and Company, USA) was flushed through the cannula to maintain patency following each blood draw. Hct, Hb, lactate, noradrenaline, and IL-6 concentration were also determined.

5.3.4. Systemic haemodynamics

Arterial blood pressure and HR were recorded continuously using a photoplethysmography device (Finometer® Pro, Finapres Medical Systems, Netherlands) throughout the experimental trials. Beat-by-beat blood pressure was obtained from participants' middle finger, and reconstructed BA pressure wave-forms were acquired after calibrating the finger pressure to BA pressure. Finometer® data was recorded using an acquisition board (PowerLab, ADInstruments, UK) and analysed using LabChart software (Labchart 6, ADInstruments, UK), where all signals were synchronised and inspected for quality. Participants' MAP was calculated by the LabChart software from the systolic and diastolic blood pressure measurements. To avoid participant discomfort, the finger cuff was removed in most participants after the 80th min measurement, and then recalibrated 10 min before the next measurement (100 min). This procedure was repeated for some participants between the 180th and 240th min measurements.

Participants' SV was calculated from apical four chambers echocardiographic measurements using a Vivid 7 ultrasound (GE, UK), as

described in the general method section. Measurements were obtained with participants lying in the semidecubitus position on their left side, which was accomplished by tilting the recumbent cycle ergometer during exercise. Echocardiographic measurements were made at the level of the 5th - 6th intercostal spaces aiming to obtain an apical four chamber view of the heart. Images were obtained using the cineloop function of the ultrasound, so a total of 20 to 30 heart cycles were recorded and the end diastolic and end systolic left ventricular area were measured and tracked using the EchoPac software (version 112, GE, UK). Left ventricular volumes (EDV and ESV) were obtained by the modified single plane Simpson's method of discs (Lang *et al.*, 2006). At least three heart cycles were selected for analysis and SV was calculated as the difference between the EDV and ESV of the left ventricle. Q̇ was the result of HR multiplied by SV, and estimated Q̇ were obtained from the Finometer® Pro in situations where the echocardiographic recordings could not be obtained or were of low quality.

5.3.5. *Limb blood flow and shear rate*

Upper and lower limb haemodynamics were assessed in resting limbs by vascular ultrasonography (Vivid 7 ultrasound, GE, UK). The left BA was assessed at rest, during leg exercise, and recovery to test whether systemic haemodynamic changes of the non-exercising limbs correlate with changes in circulating MV concentrations. The FA could not be assessed during exercise due to limitations imposed by lower limb movements. Arterial BF and vascular SR were calculated as described in the general method section, and leg BF was estimated during exercise as follow: Two leg BF = exercise Q̇ - (resting Q̇ + exercise or rest two arm BF). FA BF was estimated to be the same as baseline at 20 and 40 min of CON trial.

5.3.6. Quantification of circulating microvesicles

Circulating MVs were measured in citrated PPP by imaging flow cytometry. Briefly, after blocking samples for Fc receptor binding (Human TruStain FcX™, BioLegend, USA), anti-human CD41 and CD62E monoclonal antibodies (BioLegend, USA) were applied. Samples were incubated in the dark for 30 min and then washed once with PBS to stop binding and reduce background fluorescence. After a 10 min centrifugation step the MV pellet was resuspended in PBS and samples were measured at the slowest acquisition rate and at 60x magnification using an ImageStreamX® Mark II flow cytometer (Amnis Corporation, USA). Analysis was performed using IDEAS software (version 6.1, Amnis Corporation, USA) as described in the general methods section.

5.3.7. Plasma noradrenaline and interleukin-6 storage and analysis

Five ml of blood were added to EDTA containing tubes (Sarsterdt, Germany) and immediately centrifuged at 3,000 x g and 4°C for 10 min to obtain platelet-rich plasma. The plasma was aliquoted and stored at -80°C until analysis. Plasma noradrenaline and IL-6 concentration were measured by commercially available solid phase sandwich ELISA kits (Noradrenalin ELISA, IBL International, Germany, lot END159; Human IL-6 Quantikine, R&D Systems, USA, lot 330593) in duplicates, according to the manufacturer's instructions. Noradrenaline and IL-6 concentrations were calculated with polynomial regression equations based on standard curves after averaging optical density measurements obtained at 430 nm (for noradrenaline) and 490 nm (for IL-6) from duplicates. The mean coefficient of variation between duplicates was 9.7% for noradrenaline and 6% for IL-6.

5.3.8. Whole blood haematocrit, haemoglobin, and lactate

One ml of the blood was immediately separated in a microcentrifuge tube to determine Hct, Hb, and whole blood lactate concentration. Standard

centrifugation (HaematoSpin 1400, Hawksley, UK) of sodium-heparinized capillary tubes (micro-haematocrit tubes, Hawksley, UK) was performed to determine Hct manually, whereas Hb concentration was obtained by photometric analysis (HemoCue® Hb 201+ System, HemoCue AB, Sweden), and lactate concentration was determined using a Biosen C-line analyser (EKF Diagnostics, UK) after daily calibration.

5.3.9. Statistical analysis

All descriptive data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. Changes in the dependent variables (MV concentration, haemodynamics, and additional blood variables) were compared by two-way repeated measures ANOVA, considering Time (9 levels – from baseline to 240 min) and Condition (3 levels – CON, MI, and HI trials) as independent variables. Post hoc tests were carried out if a significant F-ratio was observed for Time effect, Condition effect, or Time x Condition interaction. The Dunnett's test for multiple comparisons was performed to compare within-condition results vs baseline values. Between-condition comparisons across each time point were performed with a separate repeated measure ANOVA, with Bonferroni correction performed manually to adjust for the number of multiple comparisons. A within-subject repeated measure correlation was performed to study the relationship between circulating MVs, haemodynamic, and biochemical variables within each participant (Bland & Altman, 1995), with data displayed as a mean group average for presentation. Stepwise multiple linear regressions were performed to identify predictors of circulating MV (dependent variable), considering BA SR, noradrenaline and IL-6 concentrations as potential independent contributors. Different from the within-subject correlation, however, multiple linear regression requires independence of observations, which was attained by performing tests for each condition and time-point (*e.g.* a model was created with data obtained at 30 minutes of heavy exercise condition). Hence data from each participant was used only once in each multiple regression model.

Due to technical issues with the GE ultrasound device, data of one individual had to be acquired using a different system (ProSound SSD5500, Aloka, Japan) during the exercise visits, with no data recorded on the control day. Due to the completely random nature of the missing data, a mean substitution treatment was applied for the missing information (in CON), instead of the listwise deletion procedure to ensure full participant datasets were incorporated in all analyses. Finally, [Hb] results could not be collected in the MI for one participant because of technical limitations, so a similar mean substitution data treatment was used.

The statistical analysis was performed using statistical software (SPSS version 20, IBM, USA), GraphPad Prism (version 5.03, GraphPad Software, USA), and SigmaPlot (version 13, Systat Software, UK) with significance level at $\alpha < 0.05$ for all analyses.

5.4. Results

5.4.1. Limb and systemic haemodynamics

Estimated lower limb BF increased during cycling as a function of exercise intensity ($P < 0.05$, Time x Condition interaction), and remained elevated at 5 min post-exercise with more pronounced values in HI reflecting the augmented metabolic demand of the lower limbs ($P < 0.05$) (Figure 5-3C). Correspondingly, early post-exercise recovery FA mean SR was higher than baseline, with substantially greater values post heavy cycling compared to moderate intensity ($P < 0.05$, Time x Condition interaction) (Table 5-2). This BF and SR response occurred mostly through a rise in blood velocity to the lower limbs ($P < 0.05$), with arterial vasodilation playing a smaller role.

During exercise, BA vasodilation occurred and the mean blood velocity was increased ($P < 0.05$) (Table 5-2), with a resultant rise in BA BF during exercise and into early recovery ($P < 0.05$, Time x Condition interaction) (Figure 5-3D). The higher BF to upper limbs during heavy compared to moderate exercise was mostly driven by changes in blood velocity, as no differences in

BA diameter were observed between exercise intensities. Similar to velocity changes, mean BA SR was augmented during cycling as a function of exercise intensity ($P < 0.05$), and was still elevated above baseline by 5 min of post heavy exercise recovery ($P < 0.05$), returning to resting values by 20 min.
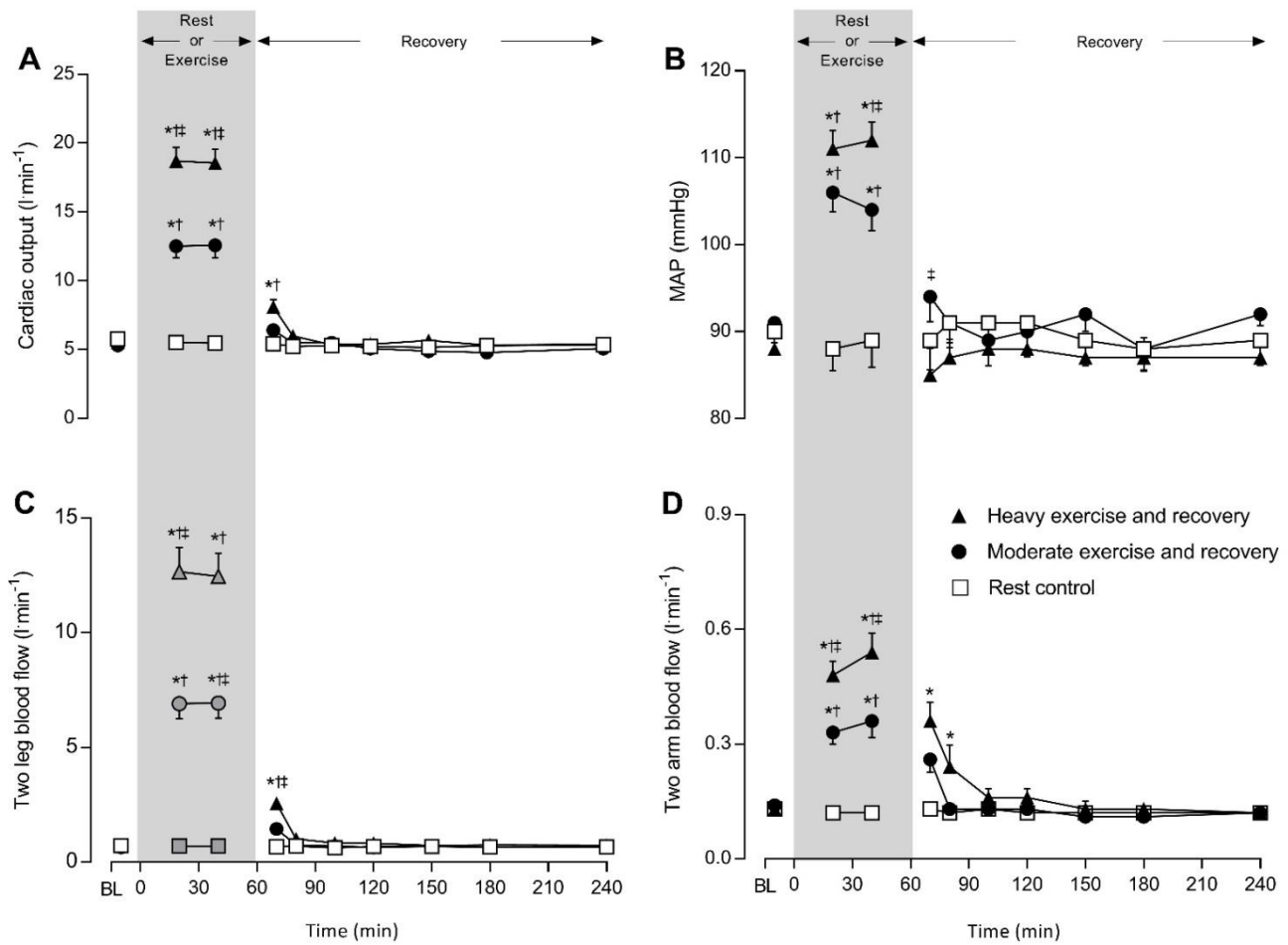
Figure 5-3. Systemic and limb haemodynamics at rest control, and during moderate, and heavy intensity exercise and subsequent recovery. Grey symbols are estimated leg blood flow. Data are mean±SEM for 9 participants. N = 7 for cardiac output. * significant different from baseline within condition ($P < 0.05$); † significant different from control visit ($P < 0.05$); ‡ significant different from moderate exercise ($P < 0.05$).

Table 5-2. Arterial and blood profile during rest control, moderate, and heavy intensity exercise visits

	Baseline	Rest or exercise		Recovery						
Time (min)	**0**	**20**	**40**	**65**	**80**	**100**	**120**	**150**	**180**	**240**
BA diameter (cm)										
Control	0.39±0.01	0.39±0.02	0.39±0.02	0.39±0.01	0.39±0.01	0.39±0.02	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01
Moderate exercise	0.39±0.01	0.41±0.01*†	0.41±0.01*†	0.40±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01
Heavy exercise	0.38±0.02	0.41±0.01*†	0.42±0.01*†	0.41±0.01*†	0.40±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.02	0.39±0.01
BA Vmean (cm·s^{-1})										
Control	9.4±0.9	8.8±0.9	8.8±1.1	9.3±1.0	8.3±1.0	8.7±0.6	8.4±0.8	8.4±0.7	8.3±0.9	8.3±1.1
Moderate exercise	10.1±1.2	19.4±2.6*$^{†#}$	20.5±3.4*$^{†#}$	16.0±2.6	10.9±2.0	9.3±1.3	8.7±1.5	8.2±1.6	7.9±1.2	7.9±1.1
Heavy exercise	10.4±1.3	30.5±2.8*†	32.3±3.3*†	23.7±3.6*†	12.9±2.5	11.6±2.0	11.5±1.9	9.3±1.6	9.9±1.4	9.0±1.3
BA mean shear rate (s^{-1})										
Control	98±10	92±10	92±13	97±13	88±13	91±8	88±10	88±10	87±11	87±13
Moderate exercise	106±15	194±28*$^{†#}$	201±36*$^{†#}$	162±29	112±21	97±21	91±18	88±19	84±15	83±14
Heavy exercise	112±18	300±32*†	309±34*†	236±40*†	135±29	122±24	120±22	97±19	106±19	97±17
FA diameter (cm)										
Control	0.90±0.02	-	-	0.89±0.02	0.90±0.02	0.89±0.02	0.89±0.02	0.89±0.02	0.89±0.02	0.90±0.02
Moderate exercise	0.90±0.02	-	-	0.96±0.02*†	0.94±0.02†	0.92±0.02	0.92±0.02	0.91±0.02	0.91±0.02	0.91±0.02
Heavy exercise	0.90±0.02	-	-	0.96±0.02*†	0.96±0.02†	0.92±0.02	0.91±0.02	0.91±0.02	0.90±0.02	0.90±0.02
FA Vmean (cm·s^{-1})										
Control	10.0±0.9	-	-	9.1±1.1	9.3±0.8	8.5±1.0	9.3±0.9	9.3±1.0	8.9±0.7	8.8±0.7
Moderate exercise	8.2±0.5	-	-	16.6±1.4*$^{†#}$	9.0±0.7	8.8±0.5	8.2±0.4	9.0±0.7	8.5±0.7	9.0±0.4
Heavy exercise	9.0±0.7	-	-	29.4±2.0*†	12.1±1.0†	10.5±0.9	10.4±0.9	9.5±0.7	9.6±0.9	9.4±0.6
FA mean shear rate (s^{-1})										
Control	45±5	-	-	42±6	42±4	38±5	42±5	42±5	41±4	40±4
Moderate exercise	37±2	-	-	70±7*$^{†#}$	39±3	36±3$^#$	36±2	40±3	38±4	40±1
Heavy exercise	40±4	-	-	122±8*†	52±5	46±5	46±4	42±4	43±4	42±3

Data are mean±SEM for 9 participants; BA, brachial artery; Vmean, time averaged mean blood flow velocity; FA, femoral artery; * $P < 0.05$ compared to baseline within condition; † $P < 0.05$ from control visit at the same time point; $^#$ $P < 0.05$ from heavy exercise visit at the same time point.

At the start of exercise HR readily increased in relation to exercise intensity and remained elevated throughout the exercise bouts ($P < 0.05$, Time x Condition interaction), returning to baseline by the 20th min of recovery after both exercise protocols, whereas SV increased during cycling independent of exercise intensity ($P < 0.05$, Time effect) and returned to resting values within the first 5 min of recovery ($P \geq 0.05$) (Table 5-3). This resulted in an elevated $\dot{Q}$ throughout cycling in accordance with exercise intensity ($P < 0.05$, Time x Condition interaction) (Figure 5-3A). In the first 5 min of recovery after heavy exercise, $\dot{Q}$ remained greater than baseline and in comparison to CON ($P < 0.05$), whereas it returned to values similar to rest following the MI protocol ($P \geq 0.05$). Similarly, MAP increased during exercise reaching higher values during the HI compared to the MI at 40 min ($P < 0.05$, Time x Condition interaction) but quickly returned to baseline at the end of exercise. The MAP at 5 min of recovery, however, was lower during the HI compared to MI reflecting a tendency for post-exercise hypotension after prolonged heavy exercise (Figure 5-3B).

Table 5-3. Cardiac parameters and blood pressure during rest control, moderate exercise, heavy exercise and subsequent recovery

	Baseline	Rest or exercise		Recovery						
Experimental conditions										
Time (min)	0	20	40	65	80	100	120	150	180	240
Heart rate (beats·min^{-1})										
Control	59±4	61±4	60±3	61±2	58±3	58±3	59±3	57±3	59±3	60±4
Moderate exercise	59±3	118±5*†#	118±6*†#	86±7*	63±3	65±3	61±3	58±2	56±2	57±2
Heavy exercise	61±3	157±5*†	158±4*†	84±3*†	73±2†	64±3	62±3	60±3	60±4	58±2
Left ventricular EDV (ml)										
Control	135±9	130±8	130±8	129±9	127±8	129±7	131±8	131±8	124±8	128±9
Moderate exercise	136±9	145±11	142±9	134±9	131±10	131±8	130±9	134±9	130±8	131±10
Heavy exercise	126±10	146±12	143±12	122±8	120±8	120±9	126±10	128±10	124±9	127±10
Left ventricular ESV (ml)										
Control	45±6	46±6	44±5	45±5	45±5	44±5	45±5	46±5	41±5	44±6
Moderate exercise	53±8	43±7	41±6*	47±6	47±6	49±6	49±7	50±6#	47±6	48±7
Heavy exercise	46±7	27±7	27±7	35±2	38±3	39±5	44±7	44±6	45±9	43±6
Stroke volume (ml)										
Control	92±4	86±3	89±4	89±4	87±4	89±3	89±4	88±3	87±3	89±4
Moderate exercise	86±3	107±6*†	107±5*†	96±5	86±3	84±2	83±3†	84±3	84±3	88±4
Heavy exercise	89±5	119±6*†	116±5*†	79±10	82±3	80±2†	82±2†	90±5	83±3†	89±5
SBP (mmHg)										
Control	130±2	127±2	127±3	127±4	129±4	129±4	128±4	124±4	123±4	126±4
Moderate exercise	130±3	160±3*†#	156±4*†#	134±3	125±2	125±4	127±4	129±3#	125±4	129±3
Heavy exercise	129±2	173±3*†	176±3*†	123±5	119±2	121±3	121±2	120±3	119±3	122±3
DBP (mmHg)										
Control	70±2	69±3	69±3	70±4	71±3	71±3	73±3	71±3	71±3	71±3
Moderate exercise	71±1	79±2*	77±2*	74±3#	73±2	72±3	72±3	73±2	70±2	74±2
Heavy exercise	68±2	80±2*	80±3*	65±2	71±1	72±2	72±2	70±2	70±2	69±2

Data are expressed in mean±SEM for 9 participants. N = 5 for cardiac volumes and 7 for stroke volume (data from 2 subjects were obtained by photoplethysmography). EDV, end diastolic volume; ESV, end systolic volume; SBP, systolic blood pressure; DBP, diastolic blood pressure; * $P < 0.05$ compared to baseline within condition; † $P < 0.05$ from control visit at the same time point; # $P < 0.05$ from heavy exercise visit at the same time point.

5.4.2. Plasma platelet and endothelial derived microvesicles

[EMV] was stable throughout the 4 h of CON and did not change in MI and HI trials ($P \geq 0.05$) (Figure 5-4). However, a significant Time x Condition interaction ($P < 0.05$) was observed for [PMV], which increased with heavy exercise. A biphasic PMV response was apparent during HI trial; increasing by ~2 fold during 30 and 60 min of heavy exercise ($P < 0.05$) after which a decrease occurred, however a second rise above baseline was evident at 60 min post heavy exercise ($P < 0.05$). Plasma volume corrections did not eliminate any changes in PMVs during and after heavy exercise ($P < 0.05$, data not show).

The PMV area under the time-concentration curve was higher during HI (154 ± 16 PMV$\cdot\mu l^{-1}\cdot 10^{3}\cdot h$) compared to CON ($89 \pm 6$ PMV$\cdot\mu l^{-1}\cdot 10^{3}\cdot h$) and MI ($108 \pm 9$ PMV$\cdot\mu l^{-1}\cdot 10^{3}\cdot h$) ($P < 0.05$, Time x Condition interaction). There were no differences in the EMV area under the curve among CON (46 ± 3 EMV$\cdot\mu l^{-1}\cdot 10^{3}\cdot h$), MI ($46 \pm 1$ EMV$\cdot\mu l^{-1}\cdot 10^{3}\cdot h$) and HI ($48 \pm 3$ EMV$\cdot\mu l^{-1}\cdot 10^{3}\cdot h$) ($P \geq 0.05$).
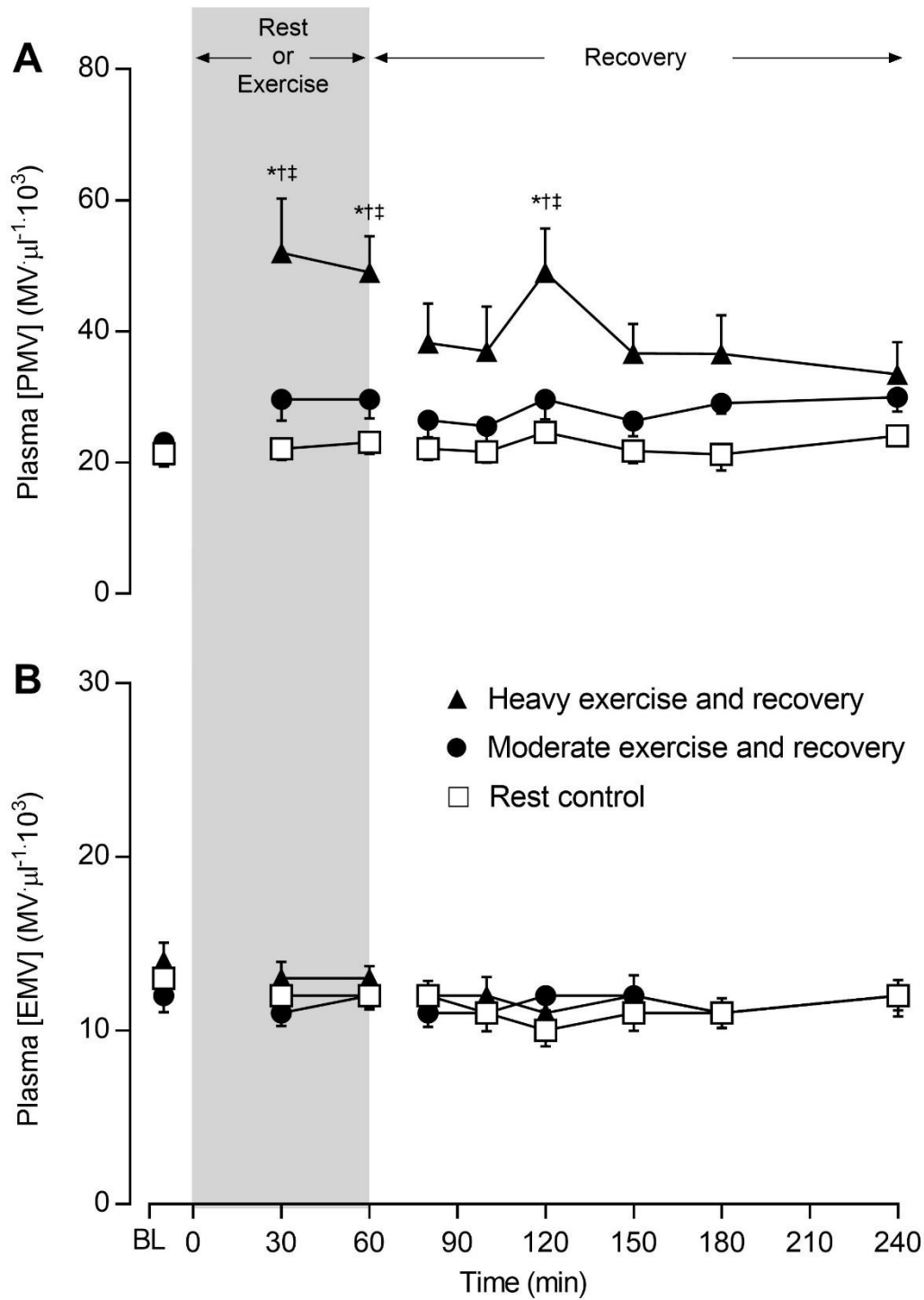
Figure 5-4. Circulating platelet (PMV - A) and endothelial (EMV - B) microvesicles at rest control, and during moderate and heavy exercise, and subsequent recovery. Exercise had no impact on [EMV], but heavy intensity cycling increased circulating PMVs during and at 1 h of post-exercise recovery. Data are mean±SEM for 9 participants. * Significant different from baseline ($P < 0.05$); † significant different from control visit ($P < 0.05$); ‡ significant different from moderate exercise ($P < 0.05$).

5.4.3. Whole-blood lactate, haemoglobin and haematocrit

Hct increased throughout the heavy intensity bout, with increases during MI evident at the end of moderate exercise (Table 5-4) ($P < 0.05$, Time x Condition interaction) and 20 min of recovery. Concomitant changes in blood Hb concentration were observed at all exercise time points during both cycling intensities ($P < 0.05$, Time x Condition interaction) and returned to baseline during recovery. Plasma volume was reduced during both moderate and heavy cycling ($P < 0.05$), and returned to baseline between 20 and 40 min after exercise ($P \geq 0.05$).

As expected heavy intensity exercise (performed above the VT1) elicited higher metabolic demands amongst the active muscles as evidenced by 5-6 fold increases in blood lactate concentrations throughout the exercise session ($P < 0.05$, Time x Condition interaction) (Table 5-4). This response was proportional to exercise intensity, with moderate exercise only inducing a small change in blood lactate (*i.e.* from ~1 at baseline to 1.6 $mmol^.l^{-1}$), although this was significant at the 30 min exercise time point ($P < 0.05$). After exercise lactate was similar to baseline ($P \geq 0.05$), but its concentration was lower throughout the first 1.5 h of recovery from moderate exercise compared to heavy ($P < 0.05$).

5.4.4. Plasma noradrenaline and interleukin-6

Plasma noradrenaline concentration rose from baseline levels during heavy cycling ($P < 0.05$, Time x Condition interaction), with greater values observed throughout the heavy cycling period compared to control, and at 30 min of exercise compared to moderate cycling ($P < 0.05$) (Table 5-4). Noradrenaline concentration returned to baseline within the first hour of recovery after heavy exercise and remained similar to baseline until the end of the recovery period ($P \geq 0.05$). No changes from baseline were observed during CON and MI ($P \geq 0.05$).

By the end of 1 h of heavy cycling IL-6 concentration increased compared to moderate intensity exercise and resting control ($P < 0.05$, Time x Condition interaction) (Table 5-4) and values remained elevated above baseline throughout the recovery period. Interestingly IL-6 concentrations also increased from baseline in the CON and MI during the 3rd and 4th h of the protocol ($P < 0.05$), so that at the end of the recovery period no differences were observed between trials.

Table 5-4. Blood-derived parameters during rest control, moderate exercise, heavy exercise and subsequent recovery

					Experimental conditions				
	Baseline	Rest or exercise		Recovery					
Time (min)	0	30	60	80	100	120	150	180	240
Haematocrit (%)									
Control	42±1	42±1	42±1	42±1	42±1	43±1	43±1	42±1	42±1
Moderate exercise	42±1	44±1	44±1*	43±1*	43±1	43±1	43±1	43±1	43±1
Heavy exercise	42±1	46±1*†	45±1*†	44±1†	43±1	43±1	43±1	43±1	43±1
Haemoglobin (g·l^{-1})									
Control	141±4	142±3	141±4	141±3	140±3	142±3	142±4	140±4	142±4
Moderate exercise	144±3	154±3*†	154±3*	146±3	146±3	146±3	146±3	148±3	145±3
Heavy exercise	142±3	155±4*†	154±4*†	147±4	141±3	139±4	142±3	142±3	142±3
PV change (%)									
Control	-	-0.4±0.7	-0.1±1.4	0.3±1.4	0.5±1.7	-1.3±1.9	-1.7±1.6	0.7±1.6	-0.9±1.4
Moderate exercise	-	-10.2±1.2*	-10.7±1.4*	-3.5±1.2	-2.8±2.5	-2.4±2.4	-2.8±2.4	-4.3±2.2	-2.2±2.1
Heavy exercise	-	-13.7±1.2*	-13.3±1.5*	-5.3±1.7*	-0.3±1.9	2.0±2.5	-0.3±1.7	-0.6±1.5	-1.1±1.3
Lactate (mmol·l^{-1})									
Control	1.0±0.1	0.9±0.1	0.9±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1
Moderate exercise	1.0±0.1	1.6±0.1*$^{#†}$	1.3±0.1$^{#†}$	0.9±0.1$^{#}$	0.8±0.1$^{#}$	0.8±0.1$^{#}$	0.7±0.1$^{#}$	0.8±0.1	1.1±0.1
Heavy exercise	1.0±0.1	6.3±0.7*†	5.1±0.9*†	2.2±0.3*†	1.4±0.2	1.2±0.1	0.9±0.1	0.9±0.1	1.0±0.1
Noradrenaline (nmol·l^{-1})									
Control	3.2±0.3	3.2±0.5	3.6±0.4	-	-	2.9±0.4	-	2.6±0.3	3.4±0.4
Moderate exercise	3.0±0.3	4.4±0.8$^{#}$	4.4±0.5	-	-	2.5±0.4	-	3.7±0.6	3.0±0.5
Heavy exercise	3.0±0.2	8.8±1.1*†	9.0±1.5*†	-	-	2.8±0.4	-	3.0±0.5	3.5±0.6
IL-6 (pg·ml^{-1})									
Control	0.8±0.1	0.8±0.2	0.7±0.2	-	-	1.1±0.3	-	2.0±0.5*	2.5±0.5*
Moderate exercise	0.8±0.2	0.7±0.1	0.9±0.1$^{#}$	-	-	1.3±0.2	-	1.7±0.2*	1.9±0.5*
Heavy exercise	0.6±0.1	0.7±0.1	1.9±0.3*†	-	-	2.0±0.4*	-	2.3±0.3*	1.8±0.1*

Data are expressed in mean±SEM for 8-9 participants; PV, plasma volume; IL-6, interleukin-6; * $P < 0.05$ compared to baseline within condition; † $P < 0.05$ from control trial at the same time point; $^{#}$ $P < 0.05$ from heavy exercise trial at the same time point.

5.4.5. Relationship between circulating microvesicles and putative agonists

Since shear stress, noradrenaline, and IL-6 have been shown to act as agonists for PMV release *in vitro*, within-subject regressions were performed to identify whether haemodynamic and biochemical variables could explain the changes observed in PMVs with exercise. A modest yet significant relationship was found between PMVs, BA SR and noradrenaline concentrations when all time-points were investigated ($P < 0.05$) (Figure 5-5A and B, respectively). Further analysis revealed that [PMV] moderately correlated with BA SR ($R^2 = 0.43$; $P < 0.05$) (Figure 5-5D) and, to a lesser degree, noradrenaline levels ($R^2 = 0.21$; $P < 0.05$) (Figure 5-5E) prior and during exercise. Furthermore, [PMV] were also related to $\dot{Q}$ ($R^2 = 0.47$; $P < 0.05$), and to estimated leg BF ($R^2 = 0.56$; $P < 0.05$) during exercise. PMVs displayed no significant correlation with IL-6 under these conditions ($P \geq 0.05$).
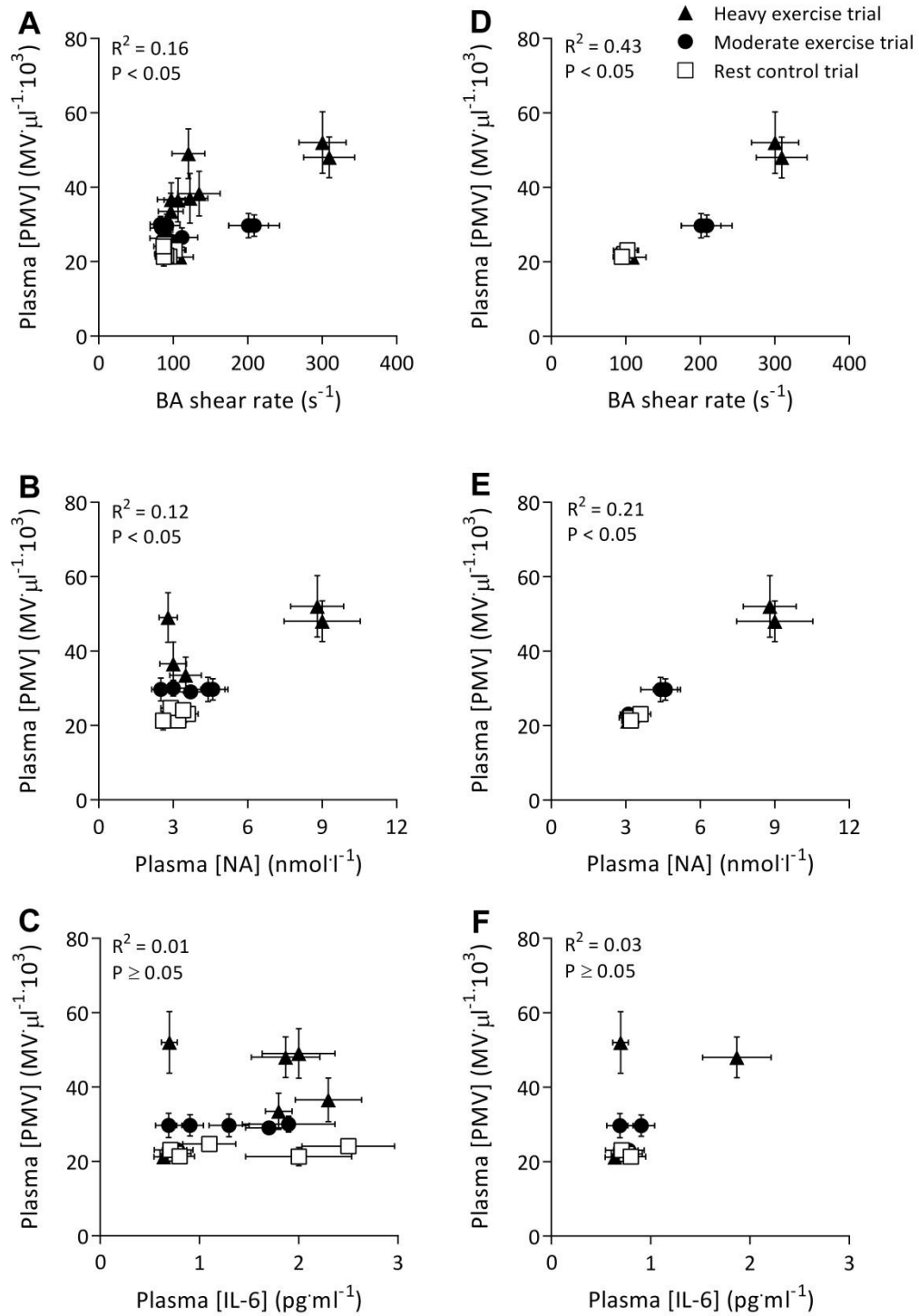
Figure 5-5. Relationship between circulating platelet microvesicle (PMV), brachial artery (BA) shear rate, noradrenaline (NA) and interleukin-6 (IL-6) concentrations at rest control, moderate, and heavy intensity exercise trials at all time-points (A-C), and only during baseline and exercise time-points (D-F). Data are mean±SEM for 9 participants.

Multiple linear regressions were further performed in an attempt to discriminate predictors of PMV changes. Using a between-subject model, the independent variables were unrelated to PMVs (Pearson Correlation; $P \geq 0.05$) at all time-points, resulting in no significant multiple linear regression model.

5.5. Discussion

This study sought to characterise the time-course of plasma MV appearance in the venous circulation during and after prolonged cycling at moderate and heavy intensities compared to a resting control condition, and to gain insights into relationships between exercise-formed MVs and potential haemodynamic and biochemical agonists *in vivo*. The main findings were that the increase in circulating PMVs, but not EMVs, occurs not only after, but also during exercise within the heavy intensity domain. Circulating [PMV] did not differ from baseline early after exercise, but a second peak was observed at 1 h post heavy cycling, suggesting a biphasic response in this MV population. Haemodynamic measurements were moderately related to the PMV dynamics during exercise, with an additional correlation observed between PMVs and plasma noradrenaline.

5.5.1. Time-course of microvesicle appearance with exercise

Little attention has been placed upon the kinetics of circulating MV appearance with exercise, with earlier efforts limited to the description of PMV dynamics during recovery. These studies generally support the concept that after exercise platelets introduce MVs into the venous circulation, with peak plasma concentrations occurring within 1-2 h of recovery (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Maruyama *et al.*, 2012). Our results demonstrate for the first time that increases in circulating PMVs are not limited to the recovery period but actually occur during exercise, and that exercise intensity influences this PMV response.

Relative exercise intensity modulates a variety of physiological responses, including increases in blood catecholamine concentrations (Galbo *et al.*, 1975; Rosenmeier *et al.*, 2004) and haemodynamic adjustments (Rådegran, 1997; Saltin *et al.*, 1998; Calbet *et al.*, 2007; Mortensen *et al.*, 2008; Chiesa *et al.*, 2015), which have been suggested to activate platelets and induce subsequent release of PMVs during exercise at high relative workrates. Another hypothesis that may explain the rise in PMVs with heavy exercise may relate to purinergic activation of platelets. As described as part of a model of haemodynamic control, exercise-induced changes in the intravascular milieu including reduced local blood oxygenation, elevated vascular shear stress, and increased blood temperature are believed to trigger the release of ATP from intra and extravascular sources in order to improve local hyperaemia (González-Alonso, 2012). Intravascular ATP can be hydrolysed to ADP and further AMP by the ectonucleoside triphosphate diphosphohydrolase, an enzyme family reported to display increased activity in platelets of exercising rats (Cardoso *et al.*, 2015). ADP has traditionally been used as an agonist in platelet function studies (Davis *et al.*, 1990; Coppola *et al.*, 2005), thus it may stimulate the generation of new PMVs. In this sense, it has been reported that acute exercise increases intravascular ADP concentrations, and this increase in circulating ADP induces platelet activation *ex vivo* (Yegutkin *et al.*, 2007). Unfortunately, quantification of circulating nucleotides was not possible in the current study, but it seems possible that likely increases in intravascular ADP may be involved in the signalling pathways leading to the formation of PMVs with exercise.

The fact that PMVs increased from baseline and then remained stable from 30 to 60 min during exercise probably indicates a rapid MV turnover, which ought to be confirmed using tracer techniques. Several processes may account for PMV removal as suggested by *in vitro* experiments. Specifically, evidence for endothelial and renal epithelial cell uptake exists (Terrisse *et al.*, 2010; Cantaluppi *et al.*, 2012). Infused endothelial progenitor cell-derived MVs were localised in renal endothelial and tubular cells of rats during recovery from renal injury, suggesting the kidney as a MV extractor organ in these conditions (Cantaluppi *et al.*, 2012). Besides the fact that MVs, including PMVs and EMVs,

have been identified in the urine of healthy humans (Viñuela-Berni *et al.*, 2015), it is still difficult to assess the fate of MVs with exercise from these experiments, even more so when one considers that renal BF (and thus MV delivery) is reduced during intense exercise (Tidgren *et al.*, 1991).

The second rise in [PMV] observed 1 h after the heavy intensity bout is intriguing, and is in agreement with other authors who found peak PMV values to occur within 1 h (Sossdorf *et al.*, 2010, 2011; Maruyama *et al.*, 2012) or 2 h (Chaar *et al.*, 2011; Sossdorf *et al.*, 2011) into the recovery period. Because these studies did not investigate circulating [PMV] at earlier recovery time points it is likely that they have missed this biphasic response, which was identified here due to the greater temporal resolution of our sampling protocol. The reason for this pattern is unknown, but implies that secondary mechanisms may lead to late platelet vesiculation after exercise since some potential platelet agonists investigated in this study, such as vascular SR, and blood noradrenaline returned to baseline levels rapidly. A potential cause of the secondary PMV peak might be related to platelet recruitment as exercise may lead not only to a pro-coagulant state (Davis *et al.*, 1990; Coppola *et al.*, 2005) but also an increase in platelet count after exercise sessions (Davis *et al.*, 1990; Ikarugi *et al.*, 1999). A reduction in plasma volume could be postulated to mediate part of this increase in platelet count through haemoconcentration but it should not be the case in the present study since plasma volume at 1 h of recovery was similar to baseline. It has also been proposed that new platelets may enter the circulation after being released from various platelet pools, including the spleen and bone marrow (El-Sayed *et al.*, 2005). Although no direct measurement was made in this study, an increase in platelet concentration could result in late MV formation by newly recruited platelets and may explain the biphasic PMV response observed in this study.

In the present experiment no change was observed in [EMV] during and after prolonged cycling, implying that the two submaximal exercise stimuli had little impact on vesiculation of endothelial cells. Inconsistent results have been published regarding EMV kinetics, with researchers reporting increases (Sossdorf *et al.*, 2011; Kirk *et al.*, 2013; Lansford *et al.*, 2015), no change (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Guiraud *et al.*,

2013; Ross *et al.*, 2014), or even decreases (Wahl *et al.*, 2014) after exercise. This apparent discrepancy is likely a result of different experimental conditions, including exercise mode, intensity and volume; and/or reflects the specific marker used to identify the EMV population. Since different proteins can be expressed by newly formed EMVs, it is plausible that the measured changes in plasma EMVs may depend on the endothelial marker investigated. For instance, Lansford et al. (2015) found increased circulating $CD62E^+$ EMVs, but not $CD42b^-/CD31^+$ EMVs, after cycling at 70% of $\dot{V}O_2max$ in men, and Kirk *et al.* (2013), reported plasma $CD105^+$ and $CD106^+$ EMVs to be elevated at 1.5 h post supramaximal cycling. Endoglin (CD105) expression is known to increase in endothelial cells undergoing angiogenesis (Nassiri *et al.*, 2011), whereas the expression of vascular adhesion molecule-1 (CD106) is upregulated in the endothelium under inflammatory stimulation. These two latter proteins, however, are also weakly expressed by other cells including monocytes and macrophages, limiting the conclusion that endothelial cells were the unique source of MVs. Considering this, we cannot rule out the hypothesis that EMVs expressing other endothelial markers could be elevated in response to exercise.

An aspect of importance in the current experiment was the description of circulating MV concentrations during CON. So far, studies investigating circulating MVs and exercise have assumed there are no temporal changes in plasma MV concentrations with natural physiological circadian variations, or due to intrinsic experimental aspects (*e.g.* issues related to successive blood drawing), accepting that any deviation from baseline was a consequence of the exercise stimulus itself (Mobius-Winkler *et al.*, 2009; Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Guiraud *et al.*, 2013; Kirk *et al.*, 2013; Ross *et al.*, 2014; Wahl *et al.*, 2014; Lansford *et al.*, 2015). The current results corroborate this idea by demonstrating that neither PMV nor EMV plasma concentrations are altered over 4 h of rest in a quiet thermoneutral laboratory environment. These control data support the validity and reproducibility of our MV concentration measures and the novel finding that [PMV] increases during heavy exercise.

5.5.2. Relationship between platelet microvesicles and putative agonists

The mechanisms eliciting PMV formation in response to exercise have not been studied by others but elevation in vascular shear stress during exercise may interact with systemic candidates to induce the observed response. It is known that as core temperature increases during exercise, a portion of the $\dot{Q}$ is distributed towards the peripheral circulation (Brengelmann *et al.*, 1977), as a consequence a rise in non-exercising limb BF during prolonged exercise may occur to facilitate dissipation of heat originating in the working muscles (Padilla *et al.*, 2011b; Simmons *et al.*, 2011). Accordingly, arm BF was elevated during exercise in the current study, and because this adjustment was driven mostly by changes in blood velocity, a concomitant rise in SR also occurred as a function of exercise intensity. As anticipated, changes in [PMV] during exercise were correlated with vascular SR, which agrees with studies where human platelets released MVs when exposed to incremental shear forces (Miyazaki *et al.*, 1996; Reininger *et al.*, 2006). A weak relationship was also apparent between noradrenaline and [PMV], which may indicate an adrenergic influence on PMV production during exercise. As previously mentioned, noradrenaline is a platelet agonist and so this association seems logical, but the precise biochemical pathways related to MV formation from noradrenaline-stimulated platelets are unknown. [PMV], however, did not correlate with IL-6 as the time course of appearance of this myokine was distinctly different from that of PMVs. These observations suggest that levels of IL-6 are not stimulating PMVs under these conditions. Supporting this is the fact that the increase in MV production reported by Nomura *et al.* (2000) occurred when incubating platelets with 100 pg.ml^{-1} of IL-6. These values far exceed the IL-6 plasma concentrations observed in this study, and thus it seems likely that physiological doses of this cytokine reached during exercise may not suffice to stimulate PMV production.

Although correlations do not necessarily represent causation, the fact that high shear stress stimulates PMV formation *in vitro*, and that plasma [PMV] changed with alterations in SR *in vivo* in the current experiment, strengthens the view that overall vascular shear stress may be one of the mediators of PMV

release during exercise. In addition, noradrenaline may play a synergistic role in stimulating PMV formation during exercise.

5.5.3. Limitations

The current experiment does have some limitations. For instance, lower limb cycling was performed whereas blood samples were obtained from an antecubital vein. Hence an assumption of this study was that peripheral venous samples mirror the MV dynamics of active limbs. Also, BA SR was used here in an attempt to characterise the importance of shear stress on MV formation. It is important to note that arm SR does not represent the magnitude of systemic shear stress generated during lower limb exercise. During cycling most of the $\dot{Q}$ is directed to the lower limbs, inevitably flowing through the descending aorta and downstream arteries. Since only small diameter changes are expected to occur in large arteries during exercise, $\dot{Q}$ measurements may seem to more reasonably reflect systemic shear stress during lower limb cycling than localised SR in the arm. Therefore, multiple linear regressions were also performed between PMVs, $\dot{Q}$, and estimated leg BF, with results indicating similar coefficients of determination compared to that observed with BA SR, further supporting the hypothesis that shear stress plays a role in the dynamics of PMV during exercise.

5.6. Conclusion

Heavy exercise increases the concentration of circulating PMVs during and at 1 h post exercise, with no impact on EMVs. This phenomenon depends on exercise intensity, since moderate cycling did not bring about this physiological response. The rise in PMVs during heavy exercise was accompanied by changes in vascular shear stress, and plasma noradrenaline concentration, and both variables appear to explain part of the MV dynamics during exercise. Therefore, this study demonstrates that exercise bouts performed in the heavy exercise domain increase the appearance of circulating

PMVs in young healthy men, potentially through the interplay of factors such as elevations in vascular shear stress and circulating catecholamines.

Chapter 6 - Study 2: Arteriovenous Microvesicle Dynamics with Heat Stress and Exercise Engaging a Large or Small Muscle Mass

6.1. Abstract

The appearance of MVs in the circulation might be a physiologically relevant response to exercise in humans but our current understanding of MV dynamics is still limited to the venous circulation, and the mechanisms underlying MV release with exercise remains largely unknown, yet increases in vascular shear stress are thought to be involved. In this study, we investigated the PMV and EMV arteriovenous (a-v) responses to increases in exercise-dependent and independent shear stress in two studies. In the first experiment, 8 trained men had vascular shear stress manipulated through passive whole-body heat stress and incremental cycling. In experiment 2, 8 additional males were exposed to passive whole-body heat stress whereas one leg remained cooled throughout, followed by dynamic single leg knee extensor exercise with the heated leg and the contralateral cooled leg. Vascular SRs in the FA were determined or estimated and blood samples were obtained from the radial artery and the femoral veins at rest and during very heavy exercise (80% PPO) in both experiments. In experiment 1, baseline [PMV] was higher in the venous than arterial circulation ($P < 0.05$). Passive heat stress increased vascular SR and arterial PMVs ($P < 0.05$) abolishing the baseline a-v PMV difference ($P \geq 0.05$). Very heavy cycling with heat stress elevated SR by ~30-fold and increased the appearance of PMVs in the venous circulation ($P < 0.05$), whereas there was no a-v PMV difference during control cycling ($P \geq 0.05$). EMVs increased from baseline in the venous circulation but only during heat stress cycling ($P < 0.05$). In experiment 2, no differences between arterial and venous samples were observed ($P \geq 0.05$) and heat stress whilst one leg remained cooled did not affect [PMV] or [EMV] ($P \geq 0.05$), despite a ~3-fold increase in vascular SR in the heated leg ($P < 0.05$). Very heavy knee extensor exercise further increased active leg SRs by ~12-fold from baseline and stimulated the release of PMVs which appeared at all sampling sites ($P < 0.05$). Taken together, these findings reveal that whole-body heat stress increases arterial [PMV], and very heavy large or small muscle mass exercise stimulates an increase in both arterial and the venous [PMV]. However, local shear stress does not appear to be a primary stimulus for localised formation of PMVs, since they also increased in the venous circulation of non-active limbs.

6.2. Introduction

The previous chapter demonstrates that the [PMV] is elevated in the circulation during heavy exercise, which may be linked to increases in vascular shear stress. Our understanding of the physiological responses underlying cellular vesiculation, however, is limited and remains restricted to events happening within the venous circulation. In the current study, we took a step further to understand the dynamics of circulating MVs in the arterial and venous circulation of exercising humans, and the potential relevance of vascular shear stress in this process.

Strenuous exercise involving a large muscle mass, such as cycling, running, or rowing, unarguably imposes very high demands upon physiological systems. During exercise, increases in blood velocity augment the laminar shear forces between the blood and its surrounding vasculature, causing shear stress to rise (Tanaka *et al.*, 2006; Padilla *et al.*, 2011b; Simmons *et al.*, 2011). Stimulation of platelets with shear forces has been found to promote PMV formation and appearance *ex vivo* (Miyazaki *et al.*, 1996; Reininger *et al.*, 2006), and the previous chapter of this thesis reported a relationship between plasma [PMV] during exercise and vascular SR in non-exercising limbs. Because intravascular shear forces increase dramatically in vessels feeding exercising muscles, it is reasonable to consider shear stress as a putative stimulus leading to platelet vesiculation during exercise. If that is the case, manipulation of shear stress in exercising humans should induce changes in circulating [PMV].

Large muscle mass exercise, however, also induces systemic changes in circulating factors that may act as agonists that induce MV formation. For example, under conditions of intense effort, adrenergic activity increases alongside relative exercise intensity leading to an exponential rise in circulating catecholamines (Galbo *et al.*, 1975; Rosenmeier *et al.*, 2004). Noradrenaline (Tschuor *et al.*, 2008), and other factors responsive to exercise, including angiotensin II (Burger *et al.*, 2011), and cytokines (Jimenez *et al.*, 2003; Abid Hussein *et al.*, 2008) have been identified as agonists that induce PMV and EMV release *in vitro*. Since the concentration of these potential agonists also

increases in the circulation during strenuous exercise, mechanistic information about the impact of shear stress on MV formation during large muscle mass exercise becomes inherently more difficult to isolate. Fortunately, dynamic knee extensor exercise can be used to address this shortcoming. This single limb exercise model has been used for decades for the investigation of local haemodynamic responses, since the exercising limb can be maintained relatively stable for ultrasound measurements (Andersen *et al.*, 1985). Exercise engaging a small muscle mass may reduce the number of physiological confounding factors involved in PMV formation by eliciting relatively smaller systemic neuroendocrine responses compared to larger muscle mass exercise (Mourtzakis *et al.*, 2004; Rosenmeier *et al.*, 2004), while inducing marked local haemodynamic adjustments (Mourtzakis *et al.*, 2004; Mortensen *et al.*, 2005; Calbet *et al.*, 2007; Mortensen *et al.*, 2008).

Vascular shear stress can also be modulated relatively independent of metabolism, albeit to a lesser degree, by manipulating body temperature. Passive heat stress imposes a significant stress to the cardiovascular system, with skin and limb tissue perfusion increasing as a function of the rise in local and core temperature, whereas arterial pressure can be preserved, or declines somewhat, simultaneously with marked elevations in $\dot{Q}$ (Minson *et al.*, 1998; Chiesa *et al.*, 2015). As a result, increases in vascular shear stress become evident during passive heat stress (Padilla *et al.*, 2011b; Simmons *et al.*, 2011) with minor effects on plasma catecholamines when passive heating is associated with localised single-limb cooling (Chiesa *et al.*, 2015), although muscle nerve adrenergic outflow increases progressively during whole-body heat stress with plasma noradrenaline spillover observed at high core temperatures (Kim *et al.*, 1979; Pearson *et al.*, 2011; Gagnon *et al.*, 2015). These unique features make passive heat stress and dynamic knee extensor exercise two pertinent approaches to test whether local increases in vascular shear stress with and without exercise stimulate increases in circulating MVs, and whether or not these elevations are a systemic or localised phenomenon.

Accordingly, the aim of the current study was to investigate the dynamics of PMV and EMV appearance with heat stress as well as large and small muscle mass exercise across major arterial and venous vessels; and to test

whether expected elevations in circulating MVs are closely associated with elevations in shear stress induced by passive heat stress and exercise. Two experiments were performed in which participants were exposed to whole-body passive heat stress, and combined heat stress and very heavy large muscle mass exercise, or passive heat stress followed by a single limb knee extensor exercise with one leg kept cool throughout the main experiment to modulate shear responses. We hypothesised that plasma PMV concentrations would increase from baseline during passive heat stress and exercise, and that this response would reflect local changes in vascular shear stress. The concentration of EMVs would remain similar to baseline with heat stress and small muscle mass exercise, while increasing slightly during very heavy cycling due to the greater exercise challenge.

6.3. Methods

6.3.1. Participants

Healthy male participants were recruited to partake in one of two experiments of this chapter. Eight trained male cyclists were recruited for experiment 1 (heat stress and cycling), and 8 healthy physically active males took part in experiment 2 (heat stress and knee extensor exercise with single leg cooling) (Table 6-1). Study design and aims were explained to each participant prior to obtaining informed written consent.

Table 6-1. Study 2 participants' characteristics

Experiment 1

Age (years)	26±6
Height (cm)	181±6
Body mass (kg)	76±9
BMI (kg·m^{-2})	23±2
$\dot{V}O_2$max (l·min^{-1})	4.5±0.3
Heat stress $\dot{V}O_2$max (l·min^{-1})	4.0±0.2
Cycling PPO (W)	371±33
Heat stress cycling PPO (W)	321±27

Experiment 2

Age (years)	25±4
Height (m)	176±4
Body mass (kg)	73±5
BMI (kg·m^{-2})	24±1
Knee extensor exercise PPO (W)	63±8

Mean±SD for 8 participants in each experiment; BMI, body mass index; $\dot{V}O_2$max, maximal oxygen uptake; HRmax, maximum heart rate; PPO, peak power output

6.3.2. Study design

This chapter describes the results of two experiments performed in collaboration with Dr SJ Trangmar (experiment 1) and Dr ST Chiesa (experiment 2), as it allowed acquisition of samples in an experimental setup that otherwise would not be possible. Original manuscripts exploring the effects of heat stress and exercise on human cardiovascular control within the same conditions are either under preparation (cycling and heat stress) or have been published elsewhere (Chiesa *et al.*, 2015). The first experiment was performed to investigate the effect of passive heat stress and very heavy large muscle mass exercise on arteriovenous PMV and EMV dynamics; whereas experiment 2 enabled us to gain insights into the influence of local haemodynamic factors on MV formation during passive heat stress and very heavy small muscle mass exercise.

6.3.2.1. Experiment 1

Trained cyclists attended the laboratory on two occasions: 1) to determine their PPO; and 2) for the main experimental visit, both of which involved upright cycling on an ergometer (Excalibur, Lode, Netherlands, Figure 6-1). Condition-specific PPO was obtained during two maximal incremental cycling tests during thermoneutral and heat stress conditions, starting at 50% of the predicted PPO, with 10% step increments every 2.5 min. Participants cycled between 70-90 rpm and the test was terminated when a cadence above 60 rpm could no longer be sustained for more than 3 continuous seconds. Gas analysis was performed breath-by-breath with a metabolic cart (Quark B^2, Cosmed, Italy). After 1 h of recovery under heat stressed conditions (outlined in Figure 6-2), a second incremental test was performed to determine the PPO with an elevated core body temperature (*i.e.* heat stress).

Figure 6-1. Upright cycle ergometer used in study 2.

During the main experimental trial participants arrived at the laboratory after their usual breakfast and were instrumented (see below). Following

baseline measurements, participants were exposed to passive whole-body heat stress by circulating hot water through a custom-made water perfused suit connected to a water circulator (Julabo F-34, Seelbach, Germany, 50°C) until their core temperature increased by approximately 1°C (~53 min). The suit covered the entire torso and legs of the participants, and the protocol elicited moderate heat stress (+ ~1°C core temperature and ~6°C in skin temperature). Then, participants performed incremental exercise under heat exposure (Figure 6-2), followed by a recovery period during which body temperature was allowed to return to baseline levels. After the recovery period a final incremental exercise protocol under thermoneutral conditions (control exercise) was performed. The incremental exercise protocol consisted of five 2.5 min incremental stages at percentages of their specific PPO, and blood samples were taken at the end of the fourth incremental stage (80% of PPO). The experimental protocol also included an incremental exercise bout after brief heat stress exposure leading to skin hyperthermia but normal core temperature; however, this segment of the protocol was not used in the assessment of MV dynamics due to logistical constraints and is thus not included in this thesis.
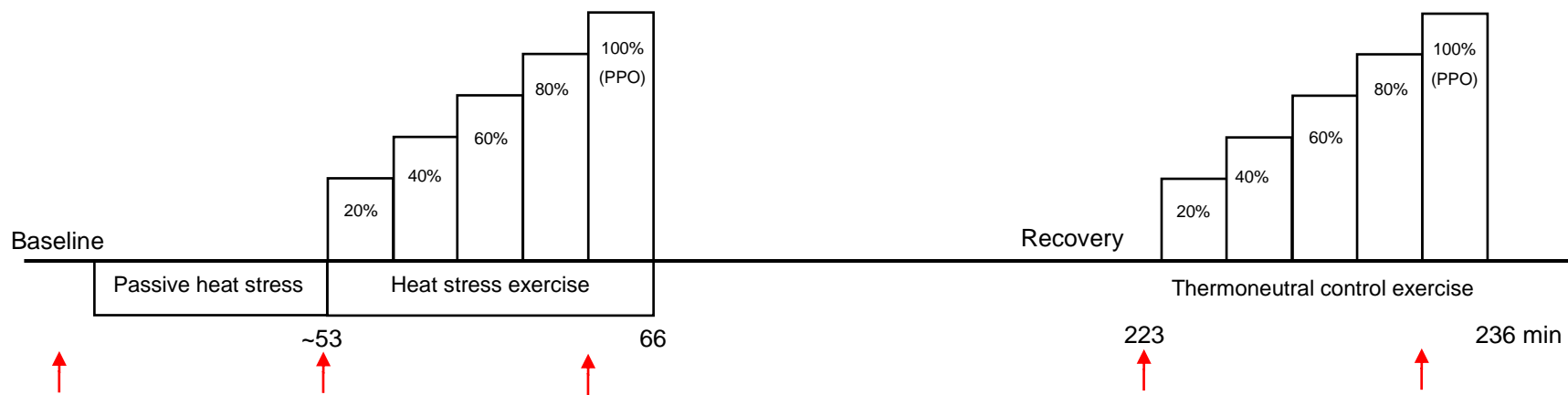
Figure 6-2. Experimental design of the upright cycling trial of study 2. Participants were exposed to whole-body passive heat stress and then incremental bouts of exercise according to their condition-specific peak power output (PPO) during heat stress and thermoneutral conditions. ⏐ blood samples.

6.3.2.2. Experiment 2

In the knee extensor experiment, participants visited the laboratory on two separate days: 1) to familiarise themselves with a custom-built single-leg knee extensor exercise ergometer and determine their individual dynamic knee-extensor exercise PPO; and 2) to perform the main experimental visit used to characterise the impact of local heat stress and exercise-induced changes in SR on arteriovenous MV dynamics. Exercise was performed on a modified Monark ergometer (Figure 6-3) with flywheel resistance controlled by friction. After familiarization with this ergometer, individual PPO of the right and left quadriceps femoris muscles was determined during an incremental test consisting of 3 min stages at 60 knee extensions per minute and 6 W increases at each stage. The test was terminated when participants could no longer maintain the cadence above 50 extensions per minute for 3 s.

Figure 6-3. Modified Monark ergometer used for the dynamic knee extensor exercise protocol in study 2.

On the day of their main experimental visit, participants had their usual breakfast and arrived at the laboratory in the morning (between 7:00 am and 9:30 am). After instrumentation (described below) and a period of rest in the supine position, baseline measurements were obtained from both legs and participants were exposed to passive heat stress for 1 h by wearing a suit (perfused with hot water, 50°C), which covered their torso and right leg, whereas the left leg was exposed to ice packs (KoolPak, Warwickshire, UK) for local limb cooling (Figure 6-4). Passive heat stress was followed by an incremental knee extensor exercise protocol performed using the cooled leg, while the heated leg remained inactive. After 20 min of recovery, the same incremental knee extensor exercise protocol was performed with the heated leg whilst the cooled limb remained inactive. Torso and right leg heating was continuously applied from the onset of passive heat stress until the end of the experimental visit (from time 0 to 104 in Figure 6-4 top), whereas the left leg was concomitantly kept cooled throughout the experimental protocols (from time 0 to 104 min in Figure 6-4 bottom).

Arterial and venous blood samples (from the heated and cooled leg) were obtained simultaneously at baseline and at the end of each condition (passive heat stress, cooled leg exercise, recovery from exercise, and heated leg exercise). Ice packs were replaced on the cooled leg every 30 min and participants drank water *ad libitum* to minimise potential confounding effects of dehydration.
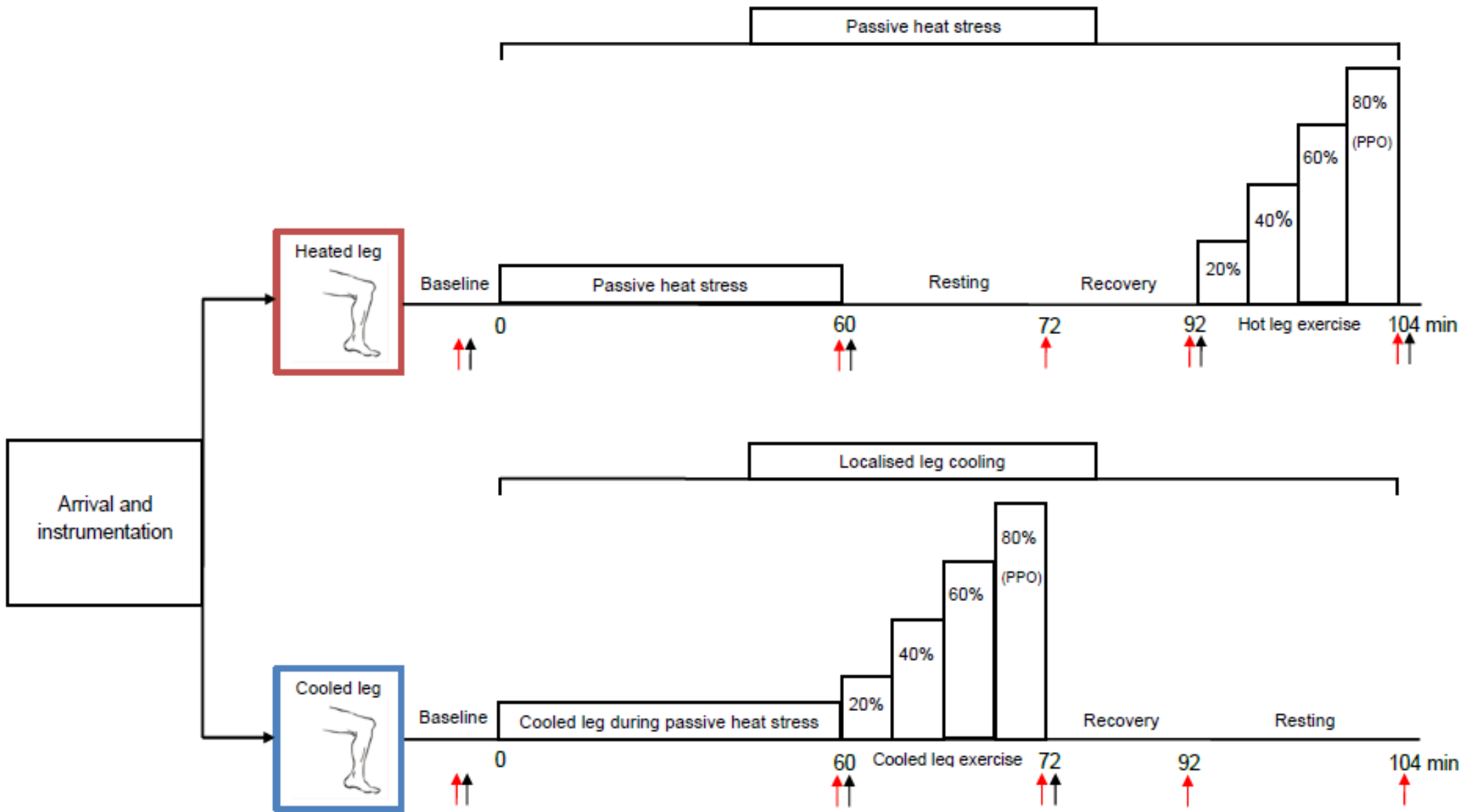
Figure 6-4. Experimental design of the single limb exercise trial of study 2. ↑ blood samples; ↑ ultrasound measurements.

6.3.3. Instrumentation and measurements

6.3.3.1. Catheter placement and blood sampling

Upon arrival, participants in both experiments were instrumented while they rested in the supine position in a thermoneutral preparation room. Arterial and venous cannulation was performed under local anaesthesia (1% lidocaine) by experienced clinicians. In experiment 1, cannulas (Logicath Quad Lumen, 18 gauge, MXA234X16X85, Smiths Medical International, UK) were placed into the non-dominant arm BA, and antegrade into the right common femoral vein using the Seldinger technique. For experiment 2, the radial artery of the right wrist was cannulated (18 gauge catheter, 16 cm, Multi-Med M2716HE, Edwards Lifesciences, USA), and intravenous cannulas were placed bilaterally in the femoral veins in the retrograde direction under ultrasound guidance with the tip of the cannulas located approximately 1 to 2 cm distal to the inguinal ligament. Blood samples were taken simultaneously from all catheters at the experimental time points indicated in Figure 6-2 and Figure 6-4. Samples were transferred to sodium citrate or EDTA blood tubes. Platelet-rich plasma was obtained by one step centrifugation, and citrated PPP was obtained by a second centrifugation process as described previously.

6.3.3.2. Circulating microvesicle quantification

Plasma MVs were measured by imaging flow cytometry in PPP obtained from citrated blood. After thawing, samples were incubated at room temperature with a Fc receptor blocking solution for 10 min (BioLegend, USA), followed by incubation with anti-human CD41 (PE/Cy5) and CD62E (PE) monoclonal antibodies (BioLegend, USA) for 30 min in the dark. After incubation, samples were washed with PBS and the pellet obtained after centrifugation was resuspended in buffer solution for imaging flow cytometric analysis using an ImageStream^X® Mark II (Amnis Corporation, USA) at 60x magnification. Quantification of MV concentrations was performed with IDEAS 6.1 software (Amnis Corporation, USA) after single-staining matrix compensation. Size gating was performed to exclude non-MV events based on exclusion of events with a diameter greater than the 1 μm diameter size calibration beads (Polysciences, USA). PMs were identified as CD41$^+$ events with

low SSC, whereas CD62E$^+$ events were used to identify EMVs, with positive events established by FMO samples. Dilution factor correction was performed to compensate for sample dilution during the acquisition analysis steps.

6.3.3.3. Blood and plasma volume changes

Participants' Hct and Hb were obtained using a blood gas and metabolite analyser (BL 800 FLEX, Radiometer, Denmark) according to the manufacturer's instructions, and the shift in blood and plasma volume was calculated as described in the general method section.

6.3.3.4. Body temperatures

In experiment 1, core body temperature was measured in the main experimental trial as blood temperature obtained from a thermistor inserted through the cannula in the common femoral vein (see Local Haemodynamics section below) and connected to a thermocouple meter (TC-2000, Sable Systems, USA). Temperature data were synchronised using an analogue-to-digital converter linked to a data acquisition board (Powerlab, ADInstruments, Australia) and data were analysed using Labchart software (version 8, ADInstruments, UK). For experiment 2, intestinal temperature was measured using a wireless telemetry temperature sensor (HQInc, Palmetto, US) which was ingested 2 to 3 h prior the main experimental trial.

Skin temperatures were obtained using wireless thermistors interfaced with data loggers (iButtons, Maxim, USA) which were attached to the skin using adhesive tape. During experiment 1, mean skin temperature was calculated as the weighted mean temperatures obtained from thermistors placed on the chest, arm, thigh and calf according to the equation: Mean Skin Temperature = 0.3 x (chest + arm) + 0.2 (thigh + calf) (Ramanathan, 1964); whereas skin temperatures in experiment 2 were obtained from thigh thermistors placed on the heated and cooled legs.

6.3.3.5. Systemic haemodynamics

In both studies, MAP was obtained from direct arterial blood pressure measurements at the brachial or radial arteries using pressure transducers positioned at the level of the heart (Pressure Monitoring Set, Edwards LifeSciences, Germany). In experiment 1, HR was obtained with a heart rate monitor (Polar Electro, Finland) and the Fick principle was used to estimate $\dot{Q}$ based on systemic $\dot{V}O_2$ and estimations of systemic O_2 extractions, as detailed in the general method section. For experiment 2, HR was calculated by the pulsatile pressure rate from the intra-arterial transducer, and SV was obtained using the Modelflow method in the radial pressure tracing with BeatScope package (FMS, Netherlands). Transducers were connected to a data amplifier (BPAmp, ADInstruments, UK) and a data acquisition system (PowerLab, AdInstruments, UK). Synchronised data analysis was performed with Labchart software (AdInstruments, UK).

6.3.3.6. Local haemodynamics

Arterial diameters and mean blood velocities within the FA were obtained by ultrasonography (Vivid 7, GE Logic, UK) using a linear array transducer for resting measurements of experiment 1 and all measurements of experiment 2. Vascular SR was calculated as 4 x time-averaged mean blood velocity / vessel diameter; and BF was the product of time-averaged mean blood velocity x π x vessel radius^2 x 60, as detailed before. The thermodilution technique was employed in experiment 1 to measure leg BF during exercise. To do so, ice cold saline was introduced into the common femoral vein through a quad lumen catheter using a Harvard pump (Harvard Apparatus, USA; ~20 s and infusion rate of 120-160 ml·min^{-1}). Changes in blood temperature were obtained using a thermistor (T204a, PhysiTemp, USA) positioned 10 cm beyond the catheter tip, after saline infusion. Leg BF values were calculated using a heat balance equation as described previously (Andersen & Saltin, 1985). Because BF values obtained by ultrasonography and thermodilution techniques are highly correlated (r = 0.996) (Rådegran, 1997), the FA vascular SR could be calculated using estimations of blood velocities from thermodilution BF, assuming relatively unchanged FA diameters as observed here and in previous

studies (Rådegran, 1997). Thus, SRs could be estimated during the cycling bouts in experiment 1.

Due to logistical constraints, haemodynamic measurements from resting legs were not recorded during the knee extensor exercise, and from the cooled leg during cooled leg recovery in experiment 2. Hence, vascular SRs in the resting leg during knee extensor exercise were estimated from BF changes, assuming that resting leg BF increased 75% during contralateral leg knee extensor exercise (Keller *et al.*, 2003). For presentation purposes, SR values in the cooled leg during recovery were assumed to be equal to those from the same leg during passive heat stress.

6.3.4. *Statistical analysis*

All descriptive data are presented as mean ± SEM, unless otherwise stated. In experiment 1, a one-way repeated measures ANOVA was used to compare differences in the dependent haemodynamic variables, and Condition (5 levels – baseline, passive heat stress, heat stress exercise, recovery, and thermoneutral control exercise, respectively). The dependent blood-derived variables, including MVs, were compared with a two-way repeated measures ANOVA, considering Condition (5 levels – from baseline to control exercise, as indicated above) and Sampling Site (2 levels – arterial and venous samples) as independent variables.

In experiment 2, change in the dependent variable vascular SR was compared with a two-way repeated measures ANOVA with Condition (5 levels – baseline, passive heat stress, cooled leg exercise, recovery, and heated leg exercise, respectively) and Leg (2 levels – heated leg and cooled leg) as independent variables; whereas Condition (5 levels – as above) and Sampling Site (3 levels – arterial, cooled leg venous, and heated leg venous samples) served as independent variables when examining changes in MVs and blood-derived variables.

Follow-up tests were carried out whenever significant F-ratios were observed across Condition, Sampling site, Leg, and when significant interactions between Condition x Sampling Site (experiment 1 and 2) or Condition x Leg (experiment 2) were observed. The Dunnett's test for multiple comparisons employed to identify

differences from baseline, and Bonferroni corrections performed *a posteriori* between conditions. Arteriovenous MV differences were compared to an expected nought value with one sample T-tests. Statistical analysis was performed using statistical software (SPSS version 20, IBM, USA) with Dunnett's *post hoc* analyses calculated using GraphPad Prism (version 5.03, GraphPad Software, USA). The significance level (α) for all tests were set at α < 0.05.

Due to technical and logistical limitations, blood samples and haemodynamic measurements could not be obtained from all participants (detailed sample sizes are displayed in the results). With regards to MVs, samples could only be obtained from 7 participants in experiment 1 and 5 participants in experiment 2. Baseline ultrasonography recordings could be obtained from 4 participants in experiment 1, with thermodilution measurements from 6 participants. Due to the completely random nature of the missing data, a mean substitution treatment was applied to estimate baseline values from ultrasound measurements, instead of the listwise deletion procedure to ensure the use of full participant datasets for analyses. During experiment 2 ultrasonography recordings from 7 individuals could be obtained, for a total of 4 participants with matching MV and local haemodynamic data sets for the within participant correlation analysis. For statistical comparisons, however, all MVs (n = 5) and local haemodynamics (n = 7) data were used.

6.4. Results

6.4.1. Experiment 1 - Responses to whole-body heat stress and very heavy cycling

6.4.1.1. Temperature and haemodynamic responses

Passive heat stress increased mean skin temperature by ~6°C (from 32.7 ± 0.4 to 38.7 ± 0.2°C), and core body temperature by ~1°C, with a further ~1.4°C increase in core temperature during cycling (P < 0.05) (Table 6-2) while skin temperature remained at ~38°C. Thermoneutral recovery decreased core body and skin temperatures, although core remained elevated above baseline (P < 0.05) and further increased during control exercise; whereas skin temperature remained at 32 ±

0.5°C. Blood [Hb] and Hct increased with passive heat stress, reflecting a reduction in participants' blood and plasma volume of ~4-8%, with further reductions observed during exercise (Table 6-2). Passive heat stress increased leg BF and SR ($P < 0.05$) (Table 6-2; Figure 6-5). During both heat stressed and control exercise, leg BF and SR increased some 30-35 fold from baseline, accompanying increases in $\dot{Q}$ and MAP ($P < 0.05$).

Table 6-2. Core temperature, haemodynamics, and haematological responses to passive heat stress and very heavy cycling

	Baseline	Passive heat stress	Heat stress exercise	Recovery	Control exercise
Core temperature (°C)	36.5±0.1	37.6±0.1*	39.0±0.1*	37.0±0.1*	38.8±0.1*
$\dot{Q}$ (l·min^{-1})	5.5±0.4	9.3±0.7*	26.1±2.6*	6.1±0.3*	25.4±1.3*
Two-leg blood flow (l·min^{-1})	0.6±0.1	2.3±0.2*	17.1±1.3*	1.5±0.4	19.2±1.0*
MAP (mmHg)	103±6	95±3	124±7*	102±4	153±7*
Haemoglobin (g·l^{-1})					
Arterial	144±3	149±3*	157±3*	139±3*	152±3*
Venous	145±3	151±3*	161±3*	140±3*	153±3*
Haematocrit (%)					
Arterial	44±1	46±1*	48±1*	43±1*	47±1*
Venous	44±1	46±1*	49±1*	43±1*	47±1*
Blood volume shift (%)					
Arterial	-	-4±1	-9±1	4±1	-6±1
Venous	-	-4±1	-10±1	3±1	-5±1
Plasma volume shift (%)					
Arterial	-	-6±1	-16±1	7±1	-10±2
Venous	-	-8±1	-18±1	6±1	-10±1

Mean±SEM for 6-7 participants. N = 4 for cardiac output. $\dot{Q}$, cardiac output; MAP, mean arterial pressure; * $P < 0.05$ compared to baseline.
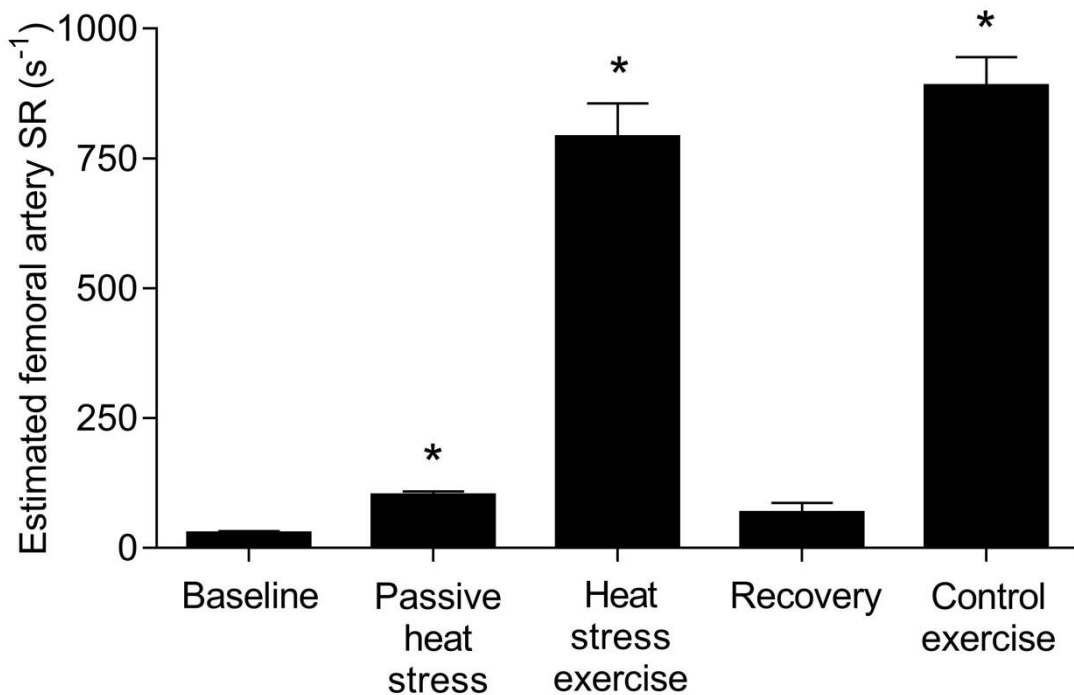
Figure 6-5. Calculated vascular shear rate (SR) in the femoral artery during baseline, passive heat stress and very heavy cycling conditions. Passive heat stress increased estimated femoral artery SR, which was further augmented during either heat stress exercise or thermoneutral control exercise. * Significant difference from baseline ($P < 0.05$).

6.4.1.2. Circulating microvesicles

Baseline concentrations of PMVs were higher in venous than arterial samples ($P < 0.05$, Condition x Sampling Site interaction) (Figure 6-6A). Passive heat stress increased the concentration of circulating arterial PMVs ($P < 0.05$), abolishing the a-v PMV difference ($P \geq 0.05$), but heat stress exercise induced substantial increases in venous PMVs, re-establishing the a-v PMV differences ($P < 0.05$) (Figure 6-6B). Arterial [PMV] remained elevated during the recovery from heat stress exercise and during control exercise ($P < 0.05$), with no difference between arterial and venous samples ($P \geq 0.05$), and venous PMVs increased at the end of the control exercise ($P < 0.05$).
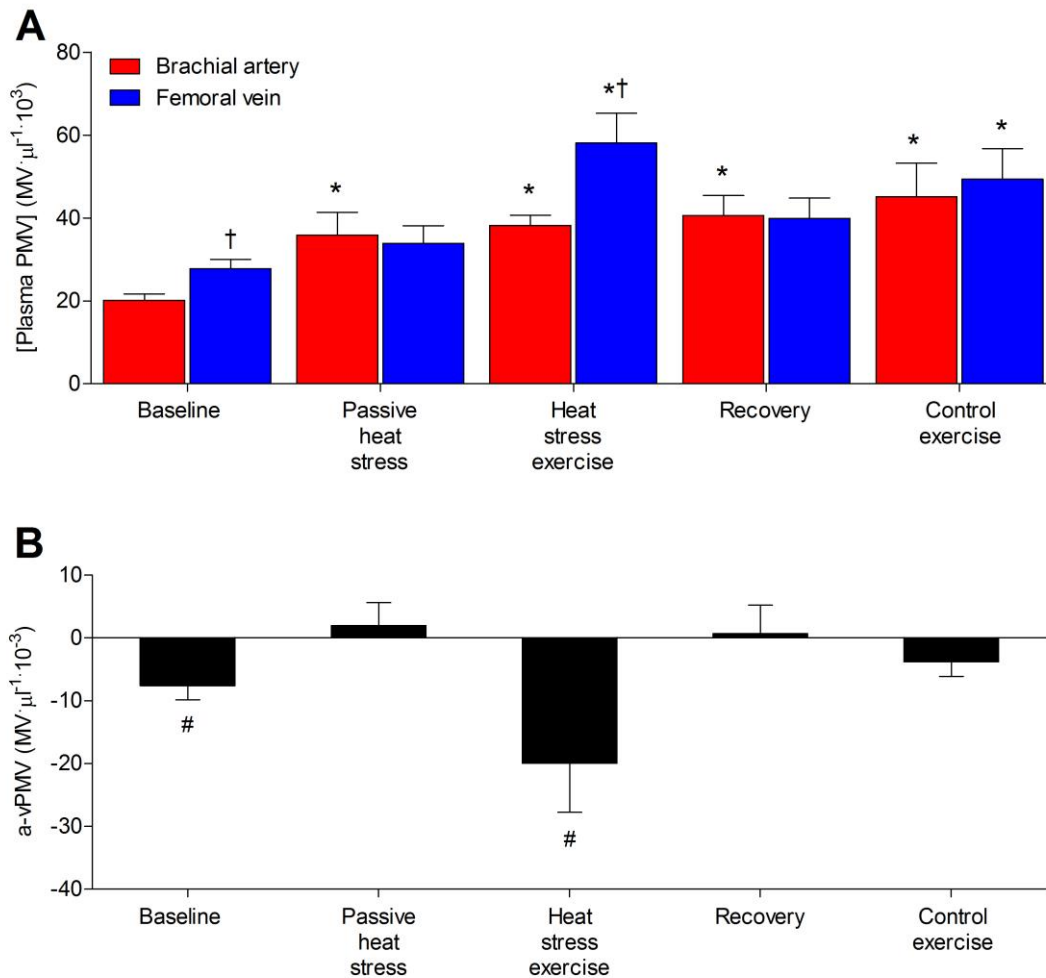
Figure 6-6. Effect of passive heat stress and very heavy cycling with and without heat stress on arterial and venous platelet microvesicle (PMV) concentrations (A), and arteriovenous PMV difference (a-vPMV) (B). Venous PMVs were higher compared to those in arterial plasma, but heat stress eliminated this difference at rest, increasing the arteriovenous difference. Exercise further stimulated PMV formation causing intravenous [PMV] to be higher than that in arterial plasma with heat stress exercise. Mean ± SEM for 7 participants. * Significant difference from baseline ($P < 0.05$); [†] significant difference compared to arterial samples at the same time-point ($P < 0.05$); [#] significant difference from zero ($P < 0.05$).

As depicted in Figure 6-7A, baseline [EMV] were similar between arterial and venous samples ($P \geq 0.05$), and passive heat stress *per se* did not affect [EMV] ($P \geq 0.05$), yet strenuous exercise combined with heat stress increased EMVs in the venous circulation compared to baseline ($P < 0.05$). Correction for plasma volume shift, however, abolished this increase [EMV] ($P \geq 0.05$). Arteriovenous EMV difference remained similar to nought throughout the study period. Venous [EMV] returned to resting levels during thermoneutral recovery, and remained similar to baseline during very heavy control exercise ($P \geq 0.05$).
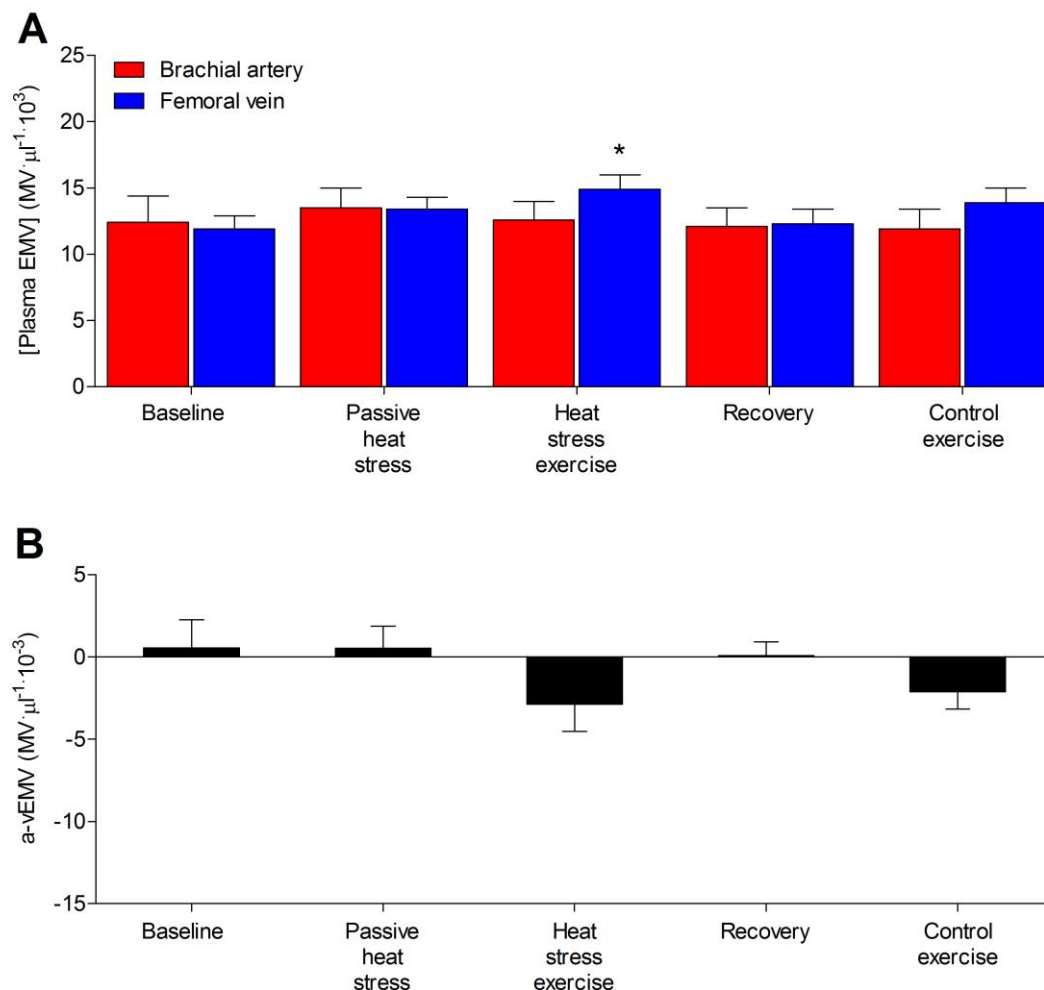
Figure 6-7. Effect of passive heat stress and very heavy cycling on arterial and venous endothelial microvesicle (EMV) concentrations (A), and arteriovenous EMV difference (a-vEMV) (B). Arterial EMVs were unaffected by passive heating and control exercise, but venous EMVs increased with cycling under heat stress, returning to baseline in during thermoneutral recovery. Mean ± SEM for 7 participants. * Significant difference from baseline ($P < 0.05$).

6.4.2. Experiment 2 – Responses to whole-body heat stress and very heavy single leg knee extensor exercise with and without local heat stress

6.4.2.1. Temperature, local and systemic haemodynamic responses

One hour of passive heat stress with localised leg cooling increased core temperature by about 0.5°C ($P < 0.05$) (Table 6-3), which remained elevated compared to baseline throughout the experimental trial. During passive heat stress, heated leg mean skin temperature increased from 28.6 ± 0.9 to 38.3 ± 1°C, whereas it decreased in the cooled leg to 17.5 ± 1.7°C ($P < 0.05$) and remained below baseline during cooled leg exercise (23.8 ± 0.6°C, $P < 0.05$). Skin temperature of the heated leg was ~8°C above baseline during heated leg exercise ($P < 0.05$). Increases in the concentration of Hb and Hct were evident at the end of cooled leg exercise, and remained elevated until the end of the trial ($P < 0.05$, Condition effect) (Table 6-3), reflecting a decrease in blood and plasma volume throughout the experimental protocol. Thermal manipulations effectively produced distinct local haemodynamic adjustments, with BFs (Table 6-3) and heated leg SRs (Figure 6-8) being higher than baseline during passive heat stress in the heated leg only ($P < 0.05$, Condition x Leg interaction) as a result of unchanged FA diameters ($P \geq 0.05$ vs baseline and between legs) but increases in FA blood velocity (from 6.7 ± 0.5 at baseline to 21 ± 2 cm·s^{-1}; $P < 0.05$). Exercise with either the cooled or the heated leg elevated their respective BFs and vascular SRs ($P < 0.05$). Passive heat stress decreased MAP ($P < 0.05$) (Table 6-3), whereas exercise with either leg elevated Q̇ and MAP ($P < 0.05$).

Table 6-3. Core temperature, haemodynamics, and haematological data during passive heat stress and very heavy single leg knee extensor exercise

	Baseline	Passive heat stress	Cooled leg Exercise	Recovery cooled leg exercise	Heated leg exercise
Core temperature (°C)	37.2±0.1	37.7±0.1*	37.8±0.1*	37.9±0.1*	38.0±0.1*
$\dot{Q}$ (l·min^{-1})	6.8±1.6	8.9±1.6*	13.5±2.2*	7.7±1.4	14.2±2.0*
Leg blood flow (l·min^{-1})					
Heated leg	0.3±0.02	0.9±0.08*†	-	0.7±0.8*	3.7±0.10*
Cooled leg	0.3±0.02	0.3±0.02	3.1±0.15*	-	-
MAP (mmHg)	107±10	91±7*	141±12*	92±16*	120±4*
Haemoglobin (g·l^{-1})					
Arterial	145±4	150±6	158±4*	155±4*	159±4*
Venous (heated leg)	144±4	149±6	160±4*	154±4*	156±4*
Venous (cooled leg)	144±4	152±5	163±4*	154±4*	158±3*
Haematocrit (%)					
Arterial	44±1	47±3	48±1*	51±3*	52±3*
Venous (heated leg)	44±1	46±2	48±1*	49±3*	49±2*
Venous (cooled leg)	44±1	47±2	50±1*	47±1*	51±3*
Blood volume shift (%)					
Arterial	-	-3±2	-8±1	-7±1	-9±1
Venous (heated leg)	-	-3±2	-10±2	-6±2	-8±2
Venous (cooled leg)	-	-5±2	-11±4	-6±2	-9±2
Plasma volume shift (%)					
Arterial	-	-8±5	-15±2	-17±5	-22±5
Venous (heated leg)	-	-6±3	-16±3	-14±5	-16±4
Venous (cooled leg)	-	-9±3	-20±3	-11±3	-20±5

Data are mean±SEM for 5-7 participants. $\dot{Q}$, cardiac output; MAP, mean arterial pressure; * $P < 0.05$ compared to baseline; † $P < 0.05$ compared to the cooled leg in the same condition.
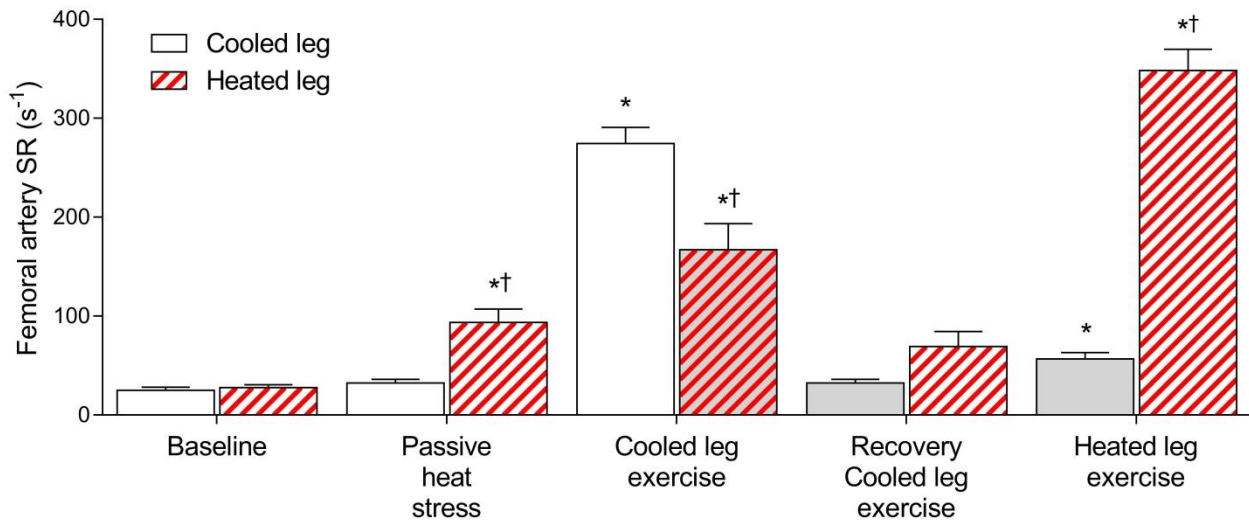
Figure 6-8. Femoral artery mean shear rate (SR) in the cooled and heated leg during passive heat stress and dynamic single leg knee extensor exercise. Grey bars are estimated SRs. Owing to changes in blood velocity, vascular SR increased in the heated leg during passive heat stress while cooling the contralateral limb with ice packs blunted these rises and diminished SR increases during exercise. Data are mean ± SEM for 7 participants. * Significant difference from baseline ($P < 0.05$); [†] significant difference compared to the cooled leg.

6.4.2.2. Circulating microvesicles

Participants' [PMV] was similar in the arterial and venous samples at rest and did not change during passive heat stress with localised one-limb cooling ($P \geq 0.5$) (Figure 6-9). However, exercise with the cooled leg increased the concentration of circulating PMVs in the arterial circulation and in both the cooled and heated leg venous circulations ($P < 0.05$, Condition effect; without Condition x Sampling Site interaction). PMVs remained elevated from baseline during recovery and heated leg exercise ($P < 0.05$). Further analysis indicated that no increase took place in circulating [PMV] from recovery to heated leg exercise ($P \geq 0.05$).
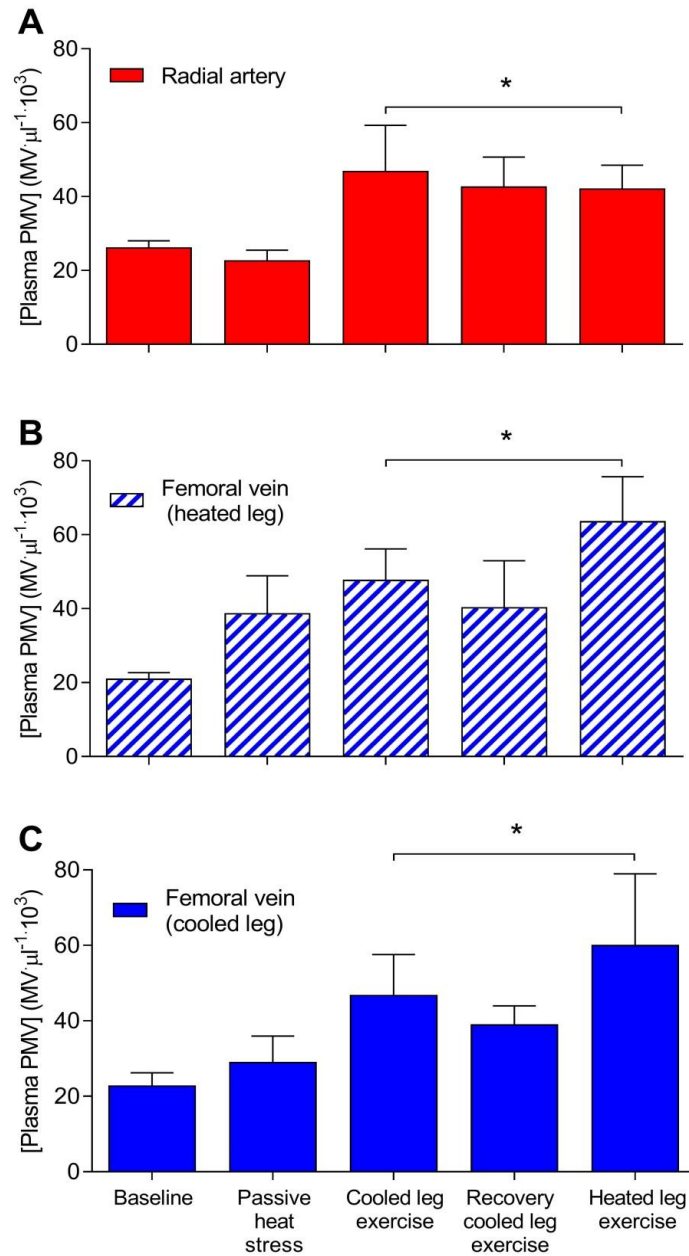
Figure 6-9. Effect of passive heat stress and dynamic single leg knee extensor exercise on arterial (A), heated leg venous (B), and cooled leg venous (C) plasma platelet microvesicles (PMVs). Heat stress did not influence plasma [PMV] at any sampling site, but PMVs increased systemically during cooled leg knee extensor exercise and remained elevated during recovery and heated leg exercise. Data are mean ± SEM for 5 participants. * Significant difference from baseline ($P < 0.05$).

Circulating [EMV] was similar in the arterial, cooled and heated leg venous samples throughout the experimental protocol ($P \geq 0.05$) (Figure 6-10). Neither passive heat stress nor dynamic knee extensor exercise with the cooled or heated leg affected the concentrations of circulating EMVs at any site of measurement ($P \geq 0.05$).
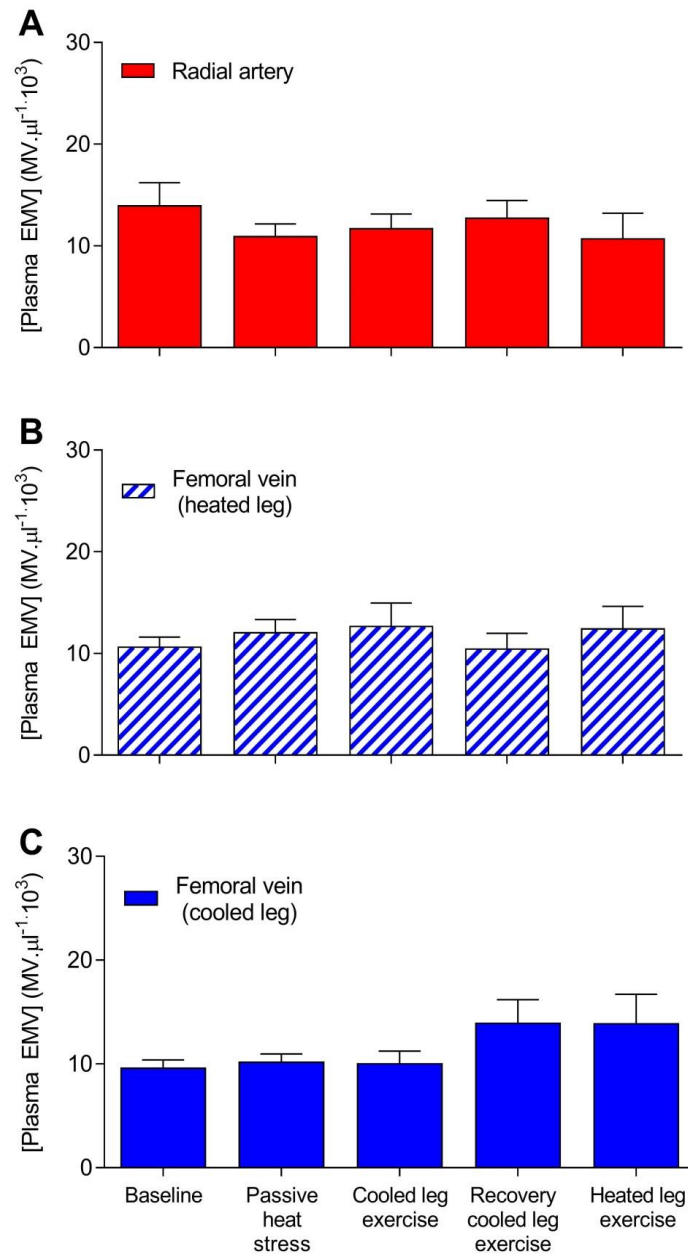
Figure 6-10. Effect of passive heat stress and dynamic single leg knee-extensor exercise on arterial (A), heated leg venous (B), and cooled leg venous (C) plasma endothelial microvesicles (EMV) concentrations. Neither heat stress nor single leg knee extensor exercise affected the [EMV]. Data are mean ± SEM for 5 participants.

6.4.3. Relationship between circulating microvesicles and shear rate

Within participant regressions revealed that arterial and venous [PMV] moderately correlated with estimations of vascular SR in the FA through haemodynamic manipulations with passive heat stress and exercise in experiment 1 ($R^2 = 0.30$, $P < 0.05$) (Figure 6-11A). In experiment 2, when arterial and venous concentrations of PMVs from the heated and cooled legs were plotted against their respective leg vascular SRs a weak, though still significant, correlation was found ($R^2 = 0.11$, $P < 0.05$) (Figure 6-11B).
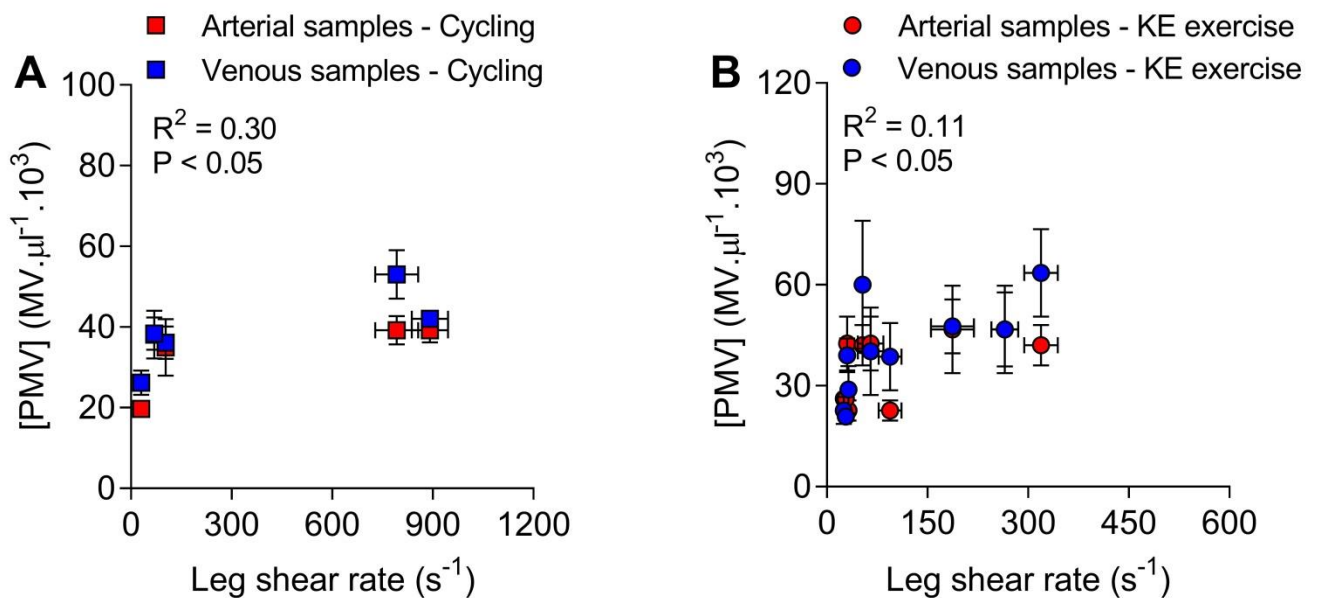
Figure 6-11. Relationship between arterial and venous platelet microvesicle (PMV) concentrations and femoral artery shear rate. Vascular shear rate was estimated during passive heat stress and cycling exercise (A), or calculated during passive heat stress with simultaneous cooling of one leg and during single limb knee extensor (KE) exercise.

6.5. Discussion

Current evidence suggests that large muscle mass exercise induces MV appearance in the venous circulation, but their arterial dynamics and the importance of local regulatory mechanisms on MV release were unknown prior to the current study. Here, we investigated the a-v PMV and EMV dynamics

during large and small muscular mass exercise to further explore the potential relevance of systemic and local vascular shear stress in MV formation. We demonstrated that the increase in PMVs with exercise was not limited to the venous circulation, and that even short duration small muscle mass exercise induces an increase in plasma [PMV]. Moreover, it appeared that the formation of PMVs in healthy humans was not under direct control of local shear stress.

6.5.1. Arteriovenous microvesicle dynamics during heat stress and exercise with small and large muscle mass

The present study investigated MV arterial and venous concentrations and a-v differences across the leg with heat stress and exercise. Notably, participants in the upright cycling trial (*i.e.* first experiment of study 2) displayed higher concentrations of PMVs in the venous circulation of the legs compared to arterial samples at baseline, suggesting PMV release across the resting leg microcirculation. Whole-body passive heat stress increased arterial [PMV] and also tended to elevate venous concentrations in these individuals. This response abolished the a-v PMV difference observed at baseline with no influence of corrections for plasma volume changes, indicating an increase in MV release from platelets. It has been reported that *in vivo* moderate passive heat stress (+1.3°C in core temperature) may increase pro-coagulant activity in the blood (Meyer *et al.*, 2013), and increased concentrations of pro-coagulant annexin-V$^+$ MVs have been reported in the circulation of baboons experiencing heatstroke induced by severe heat stress (rectal temperature ~44°C) (Bouchama *et al.*, 2008). However, *ex vivo* heat-induced platelet hyperaggregability (an index of activation) has been reported only when platelet incubation temperatures reach as high as 43°C (Gader *et al.*, 1990), suggesting that elevated temperature *per se* is not the mechanism inducing the increase in [PMV] during moderate passive heat stress seen in this thesis. Speculatively, the elevation in arterial [PMV] with passive heat stress observed in experiment 1 of this chapter may relate to purinergic activation of platelets, as an increase in plasma ATP concentration has been observed in the intra-arterial

compartment of healthy individuals exposed to moderate passive heat stress (Pearson *et al.*, 2011; Kalsi & González-Alonso, 2012).

The present study is the first to characterise the a-v MV dynamics across working limbs, and demonstrates that very heavy exercise influences the [PMV] in arterial and venous circulations. Short duration cycling elevated [PMV] in the venous circulation of the lower limbs, which is in accordance with results obtained in systemic venous samples after very heavy (Chaar *et al.*, 2011; Maruyama *et al.*, 2012) and moderate exercise (Sossdorf *et al.*, 2010, 2011). The arterial concentration of PMVs, however, remained above baseline after passive heat stress but without further evident rise during exercise (only 6% increase from passive heat stress to heated leg exercise, and 12% increase from thermoneutral recovery to control exercise). An a-v PMV difference became evident again during heat stress, resulting to greater amount of PMVs leaving the leg circulation. In contrast to large muscle mass, unilateral exercise involving a small muscle mass (*i.e.* knee extensor protocol) caused a similar increase in arterial and venous PMVs, without significant a-v differences. This rise in PMVs was apparent at all vascular sampling sites by the end of cooled leg exercise and remained elevated above baseline throughout the experimental protocol, indicating that exercise may alter MV dynamics by causing a sustained increase in PMVs during post-exercise recovery, which agrees with past investigations where blood concentrations of MVs were elevated after exercise (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011). Hence, these results demonstrate that short duration, very heavy exercise is a physiological stimulus inducing PMV formation even when a small muscle mass is involved, and indicates an increased release of PMVs into the venous circulation of exercising limbs during large muscle mass exercise.

Results concerning the appearance of PMVs in veins draining non-active limbs (*i.e.* veins in the antecubital region) during heavy cycling in the previous chapter are in agreement with the findings of the knee extensor experiment in this study, in which concomitant elevations in PMVs were observed in the venous circulation of both the active and non-active legs, and in arterial samples. These results point to a systemic effect of exercise on [PMV]. However, data from the large muscle mass exercise experiment in the current

chapter suggest that venous release of PMVs may occur in exercising limbs during heat stress incremental cycling, which seems contradictory. It is difficult to determine why a greater increase in venous compared to arterial [PMV] was observed during large muscle mass exercise during heat stress, but one potential explanation relate to the different venous sampling sites used in the experiments of chapter. In the heat stress and cycling experiment (experiment 1), catheterisation of the common femoral vein was performed in the anterograde direction, meaning that blood samples were obtained at level of the external iliac vein; whereas blood samples were obtained from the femoral vein during the knee extensor experiment (experiment 2) due to retrograde cannulation. The femoral vein receives blood from various muscular tributary vessels, whereas the external iliac vein receives blood from the femoral vein and from subcutaneous collecting vessels (*e.g.* great saphenous vein) (Black, 2014). Although evidence of specific PMV release at the level of the cutaneous circulation is lacking, it may be speculated that during combined heat stress and exercise the skin circulation may contribute to the activation of platelets, resulting in subsequent PMV release, which would explain the divergent findings observed in the current study. Further experiments, however, are necessary to shed some light upon this topic.

The fact that plasma volume corrections did not abolish the increases in [PMV] substantiates these changes as not mere artefacts caused by haemoconcentration. Instead, this raises at least three possibilities during large muscle mass exercise: an increase in PMV release, a decrease PMV uptake, or a combination of both. Increased PMV release is logical since mechanical forces (Miyazaki *et al.*, 1996; Reininger *et al.*, 2006) and biochemical agonists (Nomura *et al.*, 2000; Tschuor *et al.*, 2008) that stimulate production of PMVs are known to increase during intense exercise. Evidence supporting MV uptake also exists and derives from combined *in vitro* and *in vivo* experiments (Terrisse *et al.*, 2010; Cantaluppi *et al.*, 2012; Dasgupta *et al.*, 2012). PMV appears to undergo rapid clearance (Rand *et al.*, 2006) and might be internalised by endothelial cells in the pulmonary and systemic circulation (Terrisse *et al.*, 2010; Dasgupta *et al.*, 2012), but a reduction in PMV uptake seems less likely based

on the tendency for a greater difference between venous and arterial [PMV] during large muscle mass exercise.

The present findings also demonstrate that thermal stress coupled with large muscle mass very heavy exercise results in increases venous [EMV]. Conflicting results exist regarding changes in blood [EMV] in response to acute thermoneutral exercise, with increases (Sossdorf *et al.*, 2011; Kirk *et al.*, 2013; Lansford *et al.*, 2015), no changes (Mobius-Winkler *et al.*, 2009; Chaar *et al.*, 2011; Lansford *et al.*, 2015), and even decreases (Wahl *et al.*, 2014) in venous [EMV] reported in the literature during recovery. Interestingly, plasma volume corrections abolished the observed EMV increase in the current experiment, providing some insight about potential confounding factors related to this exercise response. As exercise starts, enhanced hydrostatic pressure drives protein-free filtrate into the extravascular compartment, which in conjunction with increasing sweating rates leads to a reduction in plasma volume (Dill & Costill, 1974). Hence, the eventual increase in plasma [EMV] does not necessarily represent endothelial activation and EMV shedding, but most likely reflects the effects of haemoconcentration. Nevertheless, surrounding cells may ultimately respond to the increase in concentration of intravascular EMVs and may respond accordingly.

6.5.2. Mechanistic insights

The formation and release of MVs from different cells is influenced by a myriad of agonists (see Table 2-1 in the Review of Literature chapter). Several platelet stimulating agents have been identified that induce PMV formation, including thrombin (Brill *et al.*, 2005), noradrenaline (Tschuor *et al.*, 2008), and interleukin-6 (Nomura *et al.*, 2000), but it remains unknown whether these *in vitro* agonists are also involved in MV formation with exercise. Vascular shear stress seems to be a potential agonist since it increases markedly with exercise (Tanaka *et al.*, 2006; Tinken *et al.*, 2009; Credeur *et al.*, 2010) and platelet stimulation with shear forces promotes PMV formation *ex vivo* (Miyazaki *et al.*, 1996; Reininger *et al.*, 2006). Since platelets are in suspension in the

circulation, increases in frictional forces between the blood and the vascular wall through rises in blood velocity also increase the shear stress on the surface of rolling platelets in contact with the endothelial layer. Regarding EMVs, however, increases in shear stress may limit endothelial vesiculation, whereas low shear forces appear to induce endothelial apoptosis and EMV release *in vitro* (Vion *et al.*, 2013b) and possibly *in vivo* (Jenkins *et al.*, 2013). In this sense, we speculated that manipulations of vascular shear stress could lead to PMV formation in humans, whereas any change in [EMV] would be linked to shear-independent mechanisms.

The haemodynamic implications of passive heat stress are diverse, with the eventual elevation in blood velocity in large arteries (Minson *et al.*, 1998; Chiesa *et al.*, 2015) leading to increases in the frictional forces between the blood and the surrounding vasculature (Tinken *et al.*, 2009; Padilla *et al.*, 2011b). In agreement with previous work, SR (a surrogate marker of shear stress) increased within the vasculature feeding heat stressed and exercising legs in both experiments of this study, whereas local cooling of the contralateral leg abolished this response. These thermal and exercise manipulations of SR provided insights on the impact of shear stress in local and systemic MV responses. Passive heat stress (+ ~0.5°C in core temperature in experiment 2) did not affect arteriovenous [PMV], although it elevated vascular SR in the heated leg to similar levels as those experienced during moderate whole-body heat stress in experiment 1. Increases in vascular SR in the cooled leg during exercise coincided with the observed increases in PMVs at all vascular sites including the contralateral resting leg, resulting in a weak association between [PMV] and vascular SR. This provides evidence against the original hypothesis of this study that local haemodynamic adjustments would influence the formation of PMVs. Nevertheless, it seems possible that a minimal level of vascular shear stress might be required to promote PMV formation *in vivo.* For example, SR in the heated leg was similar between both studies during passive heat stress (*i.e.* 3.4-fold *vs* 3.3-fold increase from baseline in experiment 1 and 2, respectively), but the overall mean vascular shear stress was inevitably lower in experiment 2 where no changes SR occurred in the cooled leg. It remains

speculative, however, whether such a systemic shear stress threshold for PMV formation really exists.

6.5.3. Limitations

Some limitations exist in the present study. By investigating each condition in a non-randomized order, we cannot exclude a possible repeated exercise effect influenced the concentration of circulating MVs in both studies. Also, factors other than haemodynamic adjustments might be involved in the MV response to exercise. Sympathetic activity, for example, increases even during mild heat stress (Gagnon *et al.*, 2015), and noradrenaline has been shown to stimulate PMV production (Tschuor *et al.*, 2008). Evidence for intravascular noradrenaline spillover with mild heat stress is equivocal (Chiesa *et al.*, 2015; Gagnon *et al.*, 2015), but it seems to occur consistently with further rises in body temperature (Kim *et al.*, 1979; Meyer *et al.*, 2013; Gagnon *et al.*, 2015). It is difficult, though, to make conclusions about adrenergic activation of platelets in the present experiments since blood catecholamine concentrations were not determined.

6.6. Conclusion

The a-v PMV and EMV dynamics in response to heat stress and exercise were investigated in the current study. We demonstrated that whole-body passive heat stress may increase arterial [PMV], while abolishing an a-v difference that resulted in a release of venous PMVs in these participants at rest. Large muscle mass exercise under heat stress restores this difference between arterial and venous samples by markedly increasing the appearance of PMVs into the venous circulation. Small muscle mass exercise also increases the concentration of PMVs, but with no differences between arterial and venous circulations, whereas blood [EMV] increase only under strenuous conditions, such as very heavy heat stress exercise involving large muscle mass, probably as a consequence of exercise-induced haemoconcentration. Finally, and

contradicting our initial hypothesis, local adjustments in vascular shear stress do not seem to be the major mechanism leading to local PMV formation with exercise, since the concentrations of these MVs were elevated even in the circulation of resting limbs where the shear stress was low. These results provide evidence for a systemic release of PMVs and raise the possibility of additional mechanisms controlling their dynamics during exercise.

Chapter 7 - Study 3: Impact of Circulating Microvesicles Produced during Exercise upon Endothelial Cells: *In Vitro* Insights

7.1. Abstract

The influence of a variety of exercise stimuli on circulating MVs have been described in this thesis, but the role played by those MVs formed during exercise remains unknown. Since exercise training brings about beneficial adaptations to the vascular endothelium, we tested the hypothesis that MVs formed during exercise would stimulate endothelial cell proliferation and angiogenesis. Circulating MVs were obtained from healthy donors (n = 6, 24 ± 1 years, 179 ± 4 cm, 83 ± 6 kg, mean ± SD) at rest and during heavy exercise and subsequently incubated in HUVEC culture to assess their effect on endothelial proliferation, migration, scratch wound-healing, and *in vitro* angiogenesis. Circulating MVs were isolated by centrifugation; those obtained at rest (rMVs) and during exercise (exMVs) were resuspended in culture medium for experiments. MV-free plasma supernatant was used as an internal control. Treatment with exMVs increased HUVEC proliferation and migration in comparison to rMV and supernatant treatments ($P < 0.05$). rMVs increased endothelial migration compared to rest supernatant treatment ($P < 0.05$), but induced similar cell proliferation when compared to exercise supernatant ($P \geq 0.05$). The exMV treatment enhanced the scratch wound closure rate by about 4 fold compared to the negative control, and had the greatest effect compared to the other treatments ($P < 0.05$). Similarly, the number of tubule-like structures and branching points was greater with exMV treatment ($P < 0.05$), although the number of branching points did not differ from rMV condition ($P \geq 0.05$). These findings demonstrate that MVs produced during exercise present a pro-angiogenic potential in cultured endothelial cells, which is linked to the proliferative and chemotactic effects of exMVs. Together, these results provide the first evidence of a novel mechanism through which exercise may mediate vascular responses.

7.2. Introduction

Since their discovery, the biological roles played by MVs have been increasingly investigated by the scientific community. A wide variety of functions have now been attributed to specific MV populations, including influencing haemostasis, inflammation, and intercellular communication (Curtis *et al.*, 2009; Sossdorf *et al.*, 2011; Tushuizen *et al.*, 2011), beyond serving as potential biomarkers that reflect the physiological state of their parent cells (Jimenez *et al.*, 2003). The isolation of MVs from single cell populations to uncover their function (*e.g.* EMVs or PMVs) is generally performed by stimulation of cell cultures or *ex vivo* platelets with specific agonists. Human fluids, such as blood, contain a mixture of MV populations (Sossdorf *et al.*, 2011; Headland *et al.*, 2014) and the isolation of a heterogeneous MV pool has frequently been performed through centrifugation protocols (Boulanger *et al.*, 2001; Vanwijk *et al.*, 2002), which allow researchers to study the impact of complex MV preparations in experimental models.

The vascular endothelium is a dynamic tissue that acts as a sensor and responds to its surrounding milieu, orchestrating a variety of functions within the cardiovascular system that vary from modulating vascular tone, to the regulation of extravascular traffic of cells and molecules (Michiels, 2003). The maintenance of adequate endothelial function is an essential step in the prevention and treatment of numerous cardiovascular diseases, with endothelial dysfunction linked to several pathological conditions and preceding poor cardiovascular outcomes (Williams *et al.*, 1996; Gokce *et al.*, 2003; Inaba *et al.*, 2010).

Endothelial cells are known to interact with circulating MVs through a variety of mechanisms that may depend on the MV source, which, in turn vary in composition based on the stimulus of formation (Boulanger *et al.*, 2001; Vanwijk *et al.*, 2002; Curtis *et al.*, 2009). As a consequence, seemingly contradictory endothelial responses may arise from MV-endothelial interactions. For example, isolated PMVs were reported to protect endothelial cells from apoptosis, and have been linked to angiogenesis (Kim *et al.*, 2004; Brill *et al.*, 2005); whereas EMVs obtained from cell cultures may promote paracrine pro-

inflammatory actions in recipient endothelial cells (Curtis *et al.*, 2009), and depress NO production *in vitro* while impairing acetylcholine dependent dilation *in situ* (Brodsky *et al.*, 2004). Moreover, plasma containing circulating MVs from myocardial infarction (Boulanger *et al.*, 2001), and pre-eclamptic patients (Vanwijk *et al.*, 2002) have been shown to depress vascular reactivity *in situ*, with no detrimental effects observed when MVs were obtained from apparently healthy donors. Although the effects of MVs upon endothelial cells have been explored using MVs isolated from single cell sources or obtained from patients, little is known about the biological function of circulating MVs produced during physiological challenges like exercise.

Exercise training has long been recognised as an efficient intervention to ameliorate the risks or even prevent cardiovascular diseases, but the protective effects of exercise against cardiovascular events are only partially explained by the related changes in traditional risk factors (Mora *et al.*, 2007), leading to the theory that non-traditional or undiscovered "exercise factors" may impart some of the observed benefits (Joyner & Green, 2009). Exercise may stimulate angiogenesis in the skeletal (Jensen *et al.*, 2004; Gavin *et al.*, 2015) and cardiac muscle (Iemitsu *et al.*, 2006), and is known to bring about increases in vascular eNOS expression (Sessa *et al.*, 1994; Laughlin *et al.*, 2001) and subsequent improvement of endothelial function through acute increases in vascular shear stress (Tinken *et al.*, 2009; Credeur *et al.*, 2010; Tinken *et al.*, 2010; Birk *et al.*, 2012). It also acts through indirect processes that may enhance NO bioavailability, such as improved anti-oxidant defences and through changes in a variety of circulating factors (Rush *et al.*, 2003; Padilla *et al.*, 2011b). Acute changes in circulatory agents have been suggested as complementary mechanisms to vascular shear stress that promote endothelial adaptations (Padilla *et al.*, 2011a), but we still have only a partial understanding of the mechanisms by which exercise training stimulates protective vascular responses. Because circulating PMVs have been shown to increase in response to exercise in previous chapters of this thesis, and this MV population has been reported to stimulate endothelial repair and morphogenesis (Kim *et al.*, 2004; Brill *et al.*, 2005), it seems plausible that circulating MVs produced during exercise may be involved in endothelial adaptation.

Therefore, the aims of this study were to test whether physiological concentrations of MVs obtained during heavy exercise would stimulate endothelial cell responses related to exercise adaptation. We hypothesised that both resting and exercise-derived MVs would enhance endothelial cell proliferation and *in vitro* angiogenesis over MV-free plasma.

7.3. Methods

7.3.1. Experimental design

To investigate the impact of exercise-derived MVs on endothelial cells, HUVECs were incubated with circulating MVs obtained at rest (rMV) and during exercise (exMV) to test their capacity to stimulate cellular migration, proliferation, wound-healing, and *in vitro* angiogenesis. Unless otherwise stated, experimental media for proliferation, and wound-healing assays were serum-free growth medium made up in M199 (wound-healing medium) or phenol red-free M199 (proliferation assay medium); whereas migration and tubule formation assays were performed in M199 with 1% penicillin-streptomycin and 0.1% HEPES. Experiments were performed by supplementing the experimental media with MVs, VEGF, or FBS as detailed in the general method section. VEGF or FBS supplemented experimental media were used as positive controls. The supernatant of MV pellets (MV-free plasma) were also used as additional internal controls.

7.3.2. Microvesicle acquisition and storage

Plasma samples for rMV and exMV isolation were obtained from male participants (Table 7-1, n = 6) who underwent 1 h of heavy exercise on a semi-recumbent ergometer as described in chapter 5 of this thesis. Citrated venous blood samples were obtained without stasis from a cannula positioned in a vein of the antecubital region at rest, and between 30 and 60 min of exercise. PPP

was obtained after the two-step centrifugation protocol and aliquots were stored at -80°C until required for experiments.

Table 7-1. Microvesicle donors' characteristics

Age (years)	24±3
Height (cm)	179±11
Body mass (kg)	83±14
BMI (kg$\cdot$m^{-2})	26±2
$\dot{V}O_2$max (l$\cdot$min^{-1})	3.3±0.7
Exercise intensity (%PPO)	63±5

Mean±SD for 6 donors; BMI, body mass index; $\dot{V}O_2$max, maximal oxygen uptake; PPO, peak power output

7.3.3. *Microvesicle supplemented medium*

On the day of the experiments, PPP vials were thawed at room temperature and aliquots transferred to new microcentrifuge tubes. Samples were diluted with the same volume of PBS and tubes were centrifuged for 1 h at 17,500 g at 4°C to pellet MVs. After centrifugation, all but 60 µl of supernatant was removed and the MV pellet was resuspended in respective experimental medium to the original concentration for the migration and wound-healing assays or to double the original MV concentration for the proliferation or tubule formation assays, but the suspension was further diluted to the original concentration in subsequent steps of those respective assays.

The plasma supernatant obtained after centrifugation was also used to make separate aliquots of MV-free experimental medium, to quantify responses to stimuli within the plasma independent of MVs that could confound observed MV effects. Accordingly, results from a pilot study, in which imaging flow cytometric analysis was performed (using the PMV marker CD41), confirmed that negligible concentrations of MVs were observed in these supernatant

samples (*i.e.* 9 ± 2% compared to the resuspended MV pellet; n = 4). Hence, the MV-free supernatant medium was used as an internal control in the current experiments.

7.3.4. Culture of human umbilical vein endothelial cells

Stored HUVECs were quickly removed from liquid nitrogen and thawed in warmed distilled water. Cells were seeded in culture flasks, slowly diluted in growth medium, and incubated at 37°C in 5% CO_2. A complete medium change was performed 24 h after seeding (Freshney, 2010b) with subsequent half medium changes every 48-72 h. Passaging or experiments were performed when monolayers became 60-80% confluent. To do so, each flask was washed with PBS, and HUVECs were detached from the coated wells by the application of trypsin. The enzyme was inactivated by FBS supplemented medium and cells were centrifuged for 6 min at 300 g. If experiments were not performed, passaging was completed by seeding the cell pellet into 3 new culture flasks with growth medium. Experiments were performed in FBS-free experimental medium unless stated otherwise, with cells between passage 3 – 6.

7.3.5. Proliferation assay

Proliferation of HUVECs was quantified by the MTT assay in pre-coated 0.5% gelatin 96-well plates in MTT experimental medium. The cell pellet was resuspended in MTT medium (FBS-free growth medium) and each well was loaded with 50 µl of the cell suspension (2,000 cells) with the same volume of conditioned experimental medium, with 20% FBS supplemented medium serving as positive control. After 48 h of incubation each well was subsequently incubated with the MTT solution for further 4 h. All but 30 µl was removed and DMSO was applied to each well before absorbance analyses using a microplate reader (ELx808, BioTek Instruments, USA). The average of quadruplicate wells was calculated and expressed as a percentage of the negative control condition.

7.3.6. Migration assay

Migration of HUVECs towards MV containing wells was measured with a modified 48 wells Boyden chamber (AP48, Neuro Probe, USA). Briefly, migration experimental medium (1% penicillin-streptomycin, 0.1% heparin M199) was loaded into wells in the lower chamber to the point of forming a slight meniscus, and a gelatin pre-coated polycarbonate filter was positioned over loaded wells. Wells loaded with 10% FBS experimental medium were used as positive control. The upper chamber was then loaded with 50 µl of a suspension of pre-starved cells (25,000 cells in 1% penicillin-streptomycin, 0.1% heparin M199), and incubated to allow cells to migrate. After disassembling the chamber, cells adherent to the migrated side of the filter were fixed with methanol and stained with Giemsa stain. Three random images of each well were obtained with an Axioskop 2 microscope (Zeiss, Germany) at 20x magnification, and the number of migrated cells were counted with ImageJ software (version 1.48, National Institutes of Health, USA). Because cells did not migrate in the FBS-free conditions, the average of triplicate wells was expressed as a percentage of 10% FBS controls.

7.3.7. Scratch wound-healing assay

The scratch wound-healing assay was performed to investigate the impact of human MVs on HUVECs undergoing wound repair. Cells were cultivated in 96-well plates pre-coated with gelatin. On the experimental day, a scratch was made on serum starved cell monolayers using a pipette tip, and cells were incubated with experimental medium. Experimental medium supplemented with 20% FBS served as positive control. Images were obtained at baseline and after incubation using an inverted microscope (Axiovert 200M, Zeiss, Germany) at 10x magnification. The ImageJ software (version 1.48, National Institutes of Health, USA) was used to determine the cell free area, from where wound closure was calculated. The average of quadruplicate wells for each condition was then expressed as a percentage of the wound closure observed in the negative control condition.

7.3.8. Tubule formation assay

The angiogenic potential of MVs was assessed by a tubule formation assay. Briefly, 96-well plates were coated with a basement membrane protein gel (Geltrex®, Thermo Fisher Scientific, UK) and incubated at 37°C to allow the gel to solidify. Starved cells were suspended in M199 (1% penicillin-streptomycin, 0.1% heparin) and 50 µl (10,000 cells) per well were incubated with the same volume of experimental medium. The plate was returned to the incubation chamber for 24 h and imaged at 5x magnification (AxioVert microscope, Zeiss, Germany). The number of tubule-like structures and branching points were determined using ImageJ software (version 1.48, National Institutes of Health, USA), and the average of triplicate wells was expressed as a percentage of tubules and branches formed in the negative control condition.

7.3.9. Statistical analysis

Results are expressed as mean ± SEM unless stated otherwise. Changes in dependent variables (*i.e.* proliferation, migration, closure of scratch wound, formation of tubule-like structures and branching points) were compared using a repeated measures ANOVA, with Condition (4 levels – rest supernatant, exercise supernatant, rMVs, and exMVs) as the independent variable. Differences between conditions were identified using the least significant difference correction when a significant F-ratio was observed. The statistical analysis was performed with SPSS software (version 20, IBM, USA), with α < 0.05.

7.4. Results

7.4.1. Proliferation

HUVECs proliferated almost 50% quicker than the negative control condition when treated with rMVs, and cell proliferation almost doubled from

control with exMVs (Figure 7-1). The exMV treatment increased HUVEC proliferation in comparison to the rMV and both the resting and exercise supernatants ($P < 0.05$). rMVs also increase HUVEC proliferation in comparison to resting supernatant, but it was similar to the exercise supernatant condition ($P \geq 0.05$). No differences in cell proliferation were observed between resting and exercise supernatants ($P \geq 0.05$).

Figure 7-1. Proliferation of human umbilical vein endothelial cells incubated with rest or exercise microvesicles, or microvesicle-free supernatant. Experimental medium supplemented with 20% foetal bovine serum (FBS) served as positive control. Top: representative images of negative control (*A*), positive control (*B*), rest (*C*) and exercise (*D*) microvesicle-free supernatant, as well as rest (*E*) and exercise (*F*) microvesicles. Bottom: Treatments with exercise microvesicles stimulated cells to proliferate at a higher rate when compared to other experimental conditions. Data are expressed in mean±SEM relative to negative control. n = 5; * significant different from rest supernatant (P < 0.05); ‡ significant different from exercise supernatant (P < 0.05); # significant different from rest microvesicles (P < 0.05).

7.4.2. *Migration*

Both MV and supernatant conditions stimulated HUVEC migration as depicted in Figure 7-2. The MV treatments, however, produced a greater stimulus for endothelial cell migration when compared with their respective supernatants ($P < 0.05$). A greater number of cells migrated towards wells loaded with exMVs in comparison to rMVs ($P < 0.05$), but no difference was observed between the two MV-free supernatant conditions.
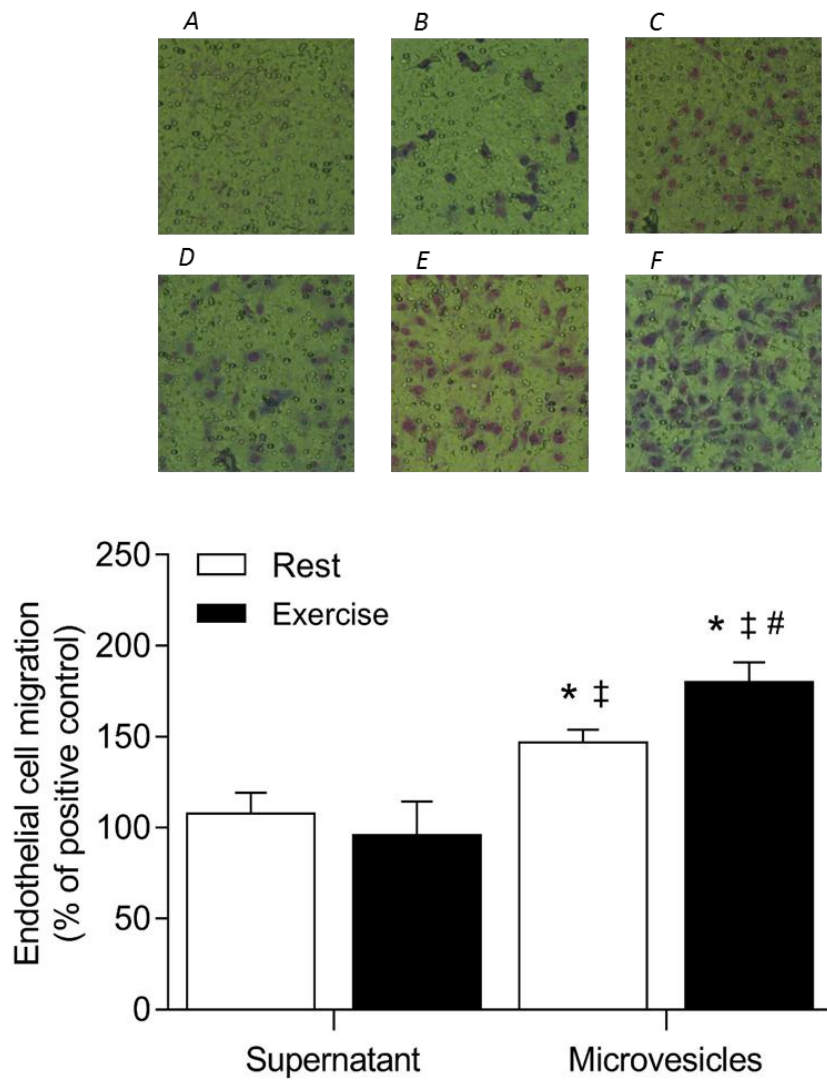
Figure 7-2. Migration of human umbilical vein endothelial cells towards experimental medium supplemented with rest or exercise microvesicles, or microvesicle-free supernatant. Top: representative images of negative control (*A*), positive control (*B*), rest (*C*) and exercise (*D*) microvesicle-free supernatant, as well as rest (*E*) and exercise (*F*) microvesicles. Bottom: Increased migration was observed towards wells loaded with exercise microvesicles in comparison to the other experimental conditions Dara are expressed in mean±SEM relative to positive control since no migration occurred towards negative control wells; n = 5; * significant different from rest supernatant ($P < 0.05$); ‡ significant different from exercise supernatant ($P < 0.05$); # significant different from rest microvesicles ($P < 0.05$).

7.4.3. Scratch wound-healing

The effects of supernatant and MV treatments were evident in the scratch wound-healing assay (Figure 7-3). HUVECs treated with supernatants displayed an almost 2-fold increase in the rate of repair compared to negative control wells, but no difference was observed between resting and exercise supernatants ($P \geq 0.05$). Both MV conditions enhanced the closure rate compared to the supernatant conditions, with rMVs stimulating wound healing to a similar extent to that observed in the FBS positive control wells (*i.e.* nearly 3 fold quicker than the negative control), and this process was augmented in the presence of exMVs ($P < 0.05$).
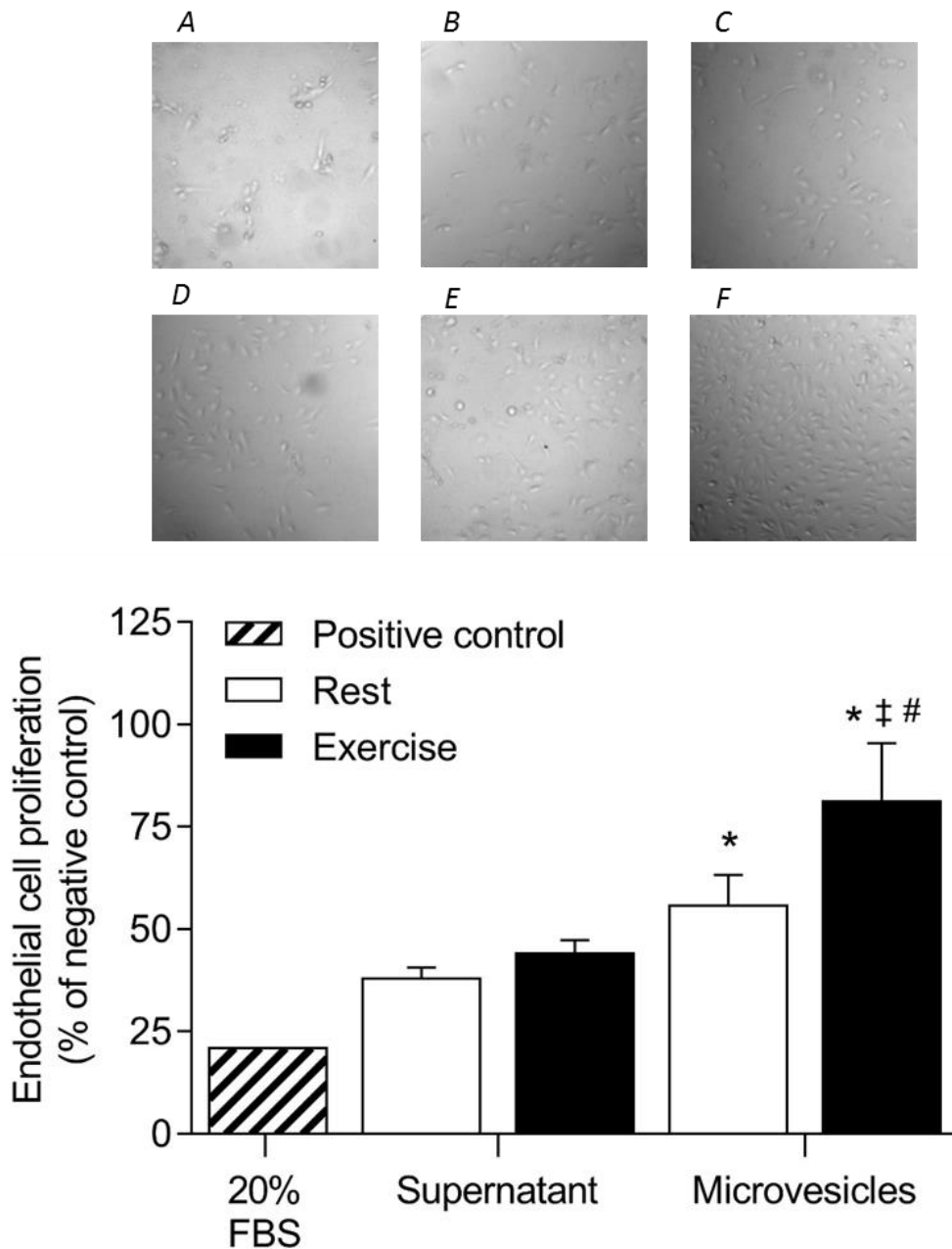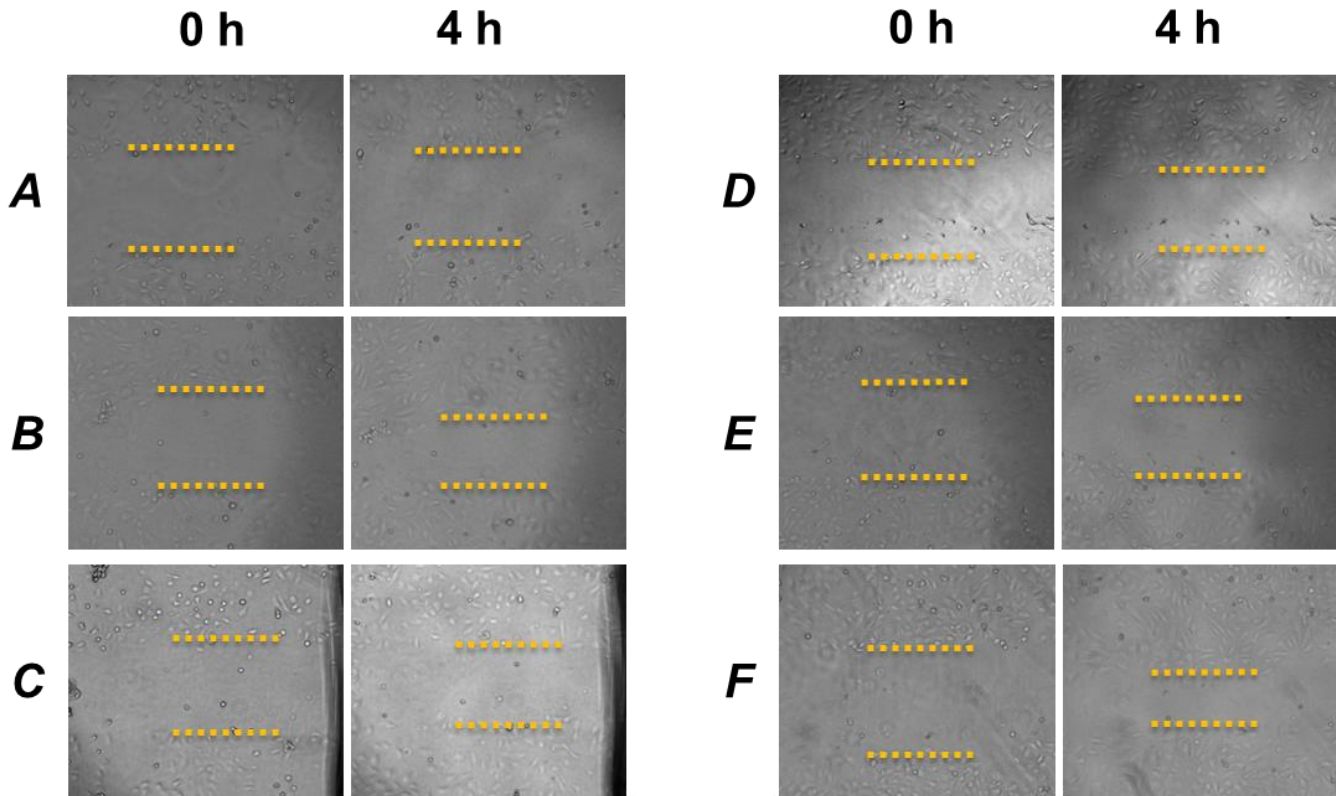
Figure 7-3. Scratch wound-healing capacity of human umbilical vein endothelial cells incubated with rest or exercise microvesicles, or microvesicle-free supernatant. Experimental medium supplemented with 20% foetal bovine serum (FBS) served as positive control. Top: representative images of negative control (*A*), positive control (*B*), rest (*C*) and exercise (*D*) microvesicle-free supernatant, as well as rest (*E*) and exercise (*F*) microvesicles. Bottom: Endothelial cells treated with exercise microvesicles displayed enhanced repair in comparison to the other experimental treatments, as measured by wound closure. Dara are expressed in mean±SEM; n = 5; * significant different from rest supernatant (P < 0.05); ‡ significant different from exercise supernatant (P < 0.05); # significant different from rest microvesicles (P < 0.05).

7.4.4. Tubule formation

HUVECs formed tubule-like structures when seeded on basement membrane matrix (Figure 7-4). Treatment with exMVs induced greater formation of tubule-like structures in comparison to all other conditions ($P < 0.05$) (Figure 7-4A), with no differences observed amongst rMVs and supernatant treatments ($P \geq 0.05$). The number of branching points was also increased in endothelial cells treated with exMVs compared to those incubated with MV-free supernatant ($P < 0.05$) (Figure 7-4B), although exMVs treatment did not increase the number of branching points in comparison to rMVs ($P \geq 0.5$).
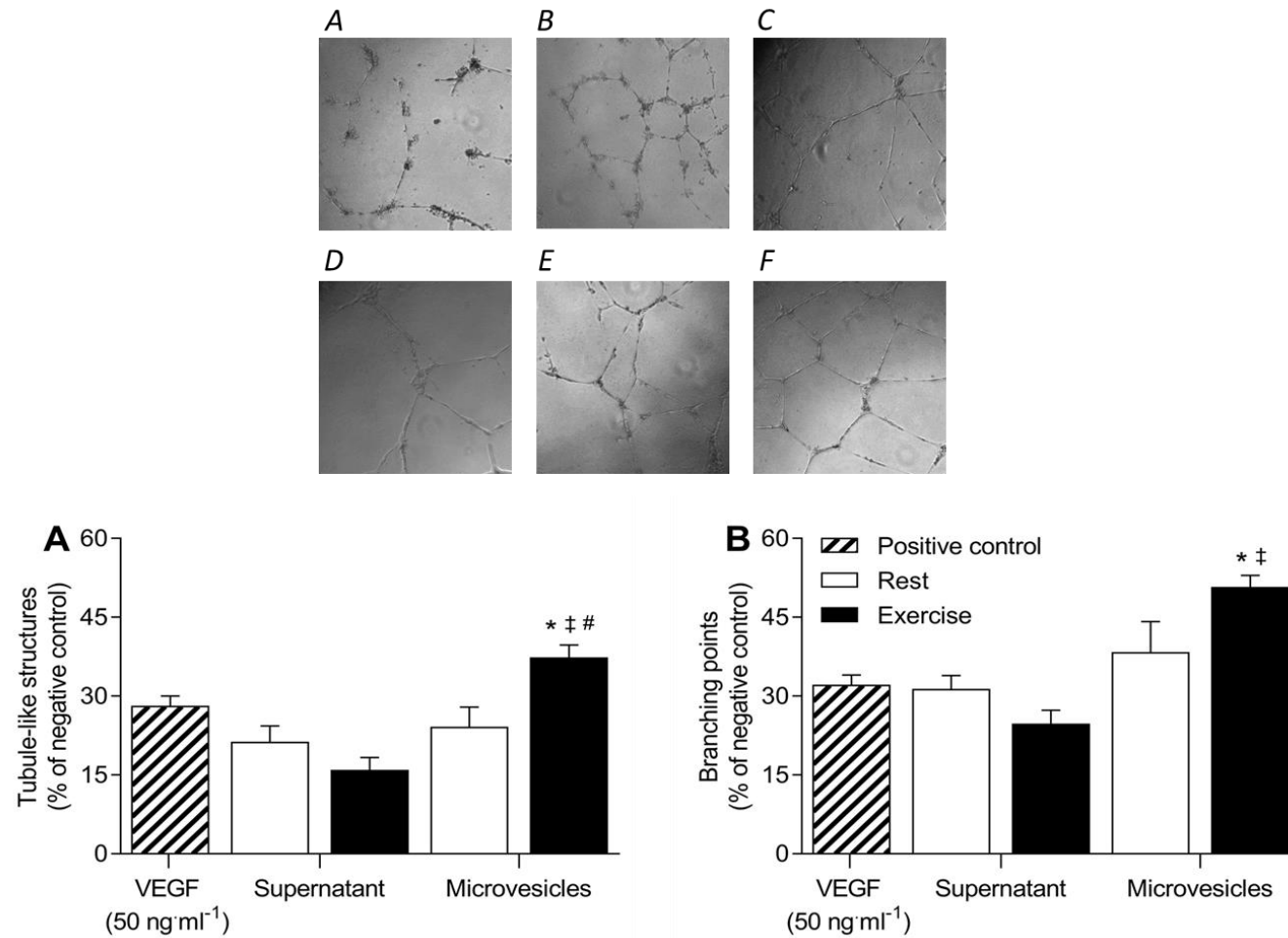
Figure 7-4. *In vitro* angiogenic parameters of human umbilical vein endothelial cells treated with rest or exercise microvesicles, or microvesicle-free supernatant. Vascular endothelial growth factor (VEGF) was used as a positive control. Top: representative images of negative control (*A*), positive control (*B*), rest (*C*) and exercise (*D*) microvesicle-free supernatant, as well as rest (*E*) and exercise (*F*) microvesicles. Bottom: exercise microvesicles increased the formation of tubule-like structures (A) in comparison to rest microvesicles and microvesicle-free supernatants; as well as the number of branching points (B) in comparison supernatant treatments. Dara are expressed in mean ± SEM; n = 5; * significant different from rest supernatant ($P < 0.05$); ‡ significant different from exercise supernatant ($P < 0.05$); # significant different from rest microvesicles ($P < 0.05$).

7.5. Discussion

Intravascular MVs have been shown to display either stimulatory (Kim *et al.*, 2004; Brill *et al.*, 2005), or inhibitory (Boulanger *et al.*, 2001; Vanwijk *et al.*, 2002; Brodsky *et al.*, 2004; Curtis *et al.*, 2009) effects on the vascular endothelium, depending on the MV cell source as well as their mechanism of formation, and heavy exercise was shown to increase [PMV] in plasma of healthy humans in this thesis, but the biological role of these submicron vesicles produced during exercise was unknown. Here, we took the first step in understanding the effects produced by MVs formed during exercise upon the vascular system, demonstrating that exMVs display a stimulatory effect in cultured endothelial cells by supporting repair and angiogenesis.

Part of the cardiovascular benefits of exercise training are mediated through endothelial adaptations in response to repetitive increases in vascular shear stress (Tinken *et al.*, 2009; Credeur *et al.*, 2010; Tinken *et al.*, 2010; Birk *et al.*, 2012), although complementary mechanisms involving the release of circulating mediators have also been hypothesised (Padilla *et al.*, 2011a). In agreement with the latter hypothesis, our experiments suggest that circulating MVs from healthy humans may be involved in the endothelial adaptive responses to exercise, since physiological concentrations of exMVs stimulated cultured endothelial cells to a greater extent than rMVs, and because the observed effects were generally blunted in treatments involving MV-free supernatant.

Cell division and migration are fundamental aspects of cellular function, and are required during development of complex organisms. In adults, endothelial cells are kept in a quiescent state but retain their proliferative capabilities and may re-enter the gap 1 phase and resume the cell cycle if required (Cooper & Hausman, 2009; Freshney, 2010a). Under favourable environmental conditions and upon growth factor stimulation endothelial cells can proliferate, and migrate toward chemotactic agents (Jaffe *et al.*, 1973b; Kim *et al.*, 2004; Kitamura *et al.*, 2008; Battinelli *et al.*, 2011). Endothelial chemotaxis and angiogenesis are part of an integrated system involving the sequential recruitment of specific signalling cascades that include the phosphoinositide 3 kinase, and mitogen activated protein kinase pathways, which act downstream of VEGF receptor activation and are critical for

endothelial cell extension and migration (Lamalice *et al.*, 2007). The findings of the present study indicate that MVs produced during exercise induce enhanced proliferative and migratory behaviour in endothelial cells compared with MVs produced during basal conditions. Findings from isolated proliferation and migration assays are supported by results of the scratch wound-healing assay, which represents a more complex process that depends on cell migratory and proliferative capacities. The fact that rMVs did not stimulate HUVEC proliferation to a greater extent than MV-free plasma suggests that resting plasma of healthy humans contains enough nutrients to support endothelial maintenance, with circulating MVs playing a minor role in this process.

Endurance training has been reported to induce angiogenesis in active tissues in animal (Iemitsu *et al.*, 2006) and human models (Jensen *et al.*, 2004; Gavin *et al.*, 2015). Angiogenesis is the complex integrated phenomenon of new capillary formation, stimulated by several stimuli including hypoxia (Deveci *et al.*, 2002) and local vascular shear stress (Galie *et al.*, 2014), with VEGF believed to be the major, albeit not single, growth factor implicated in the formation of the new capillary network (Jensen *et al.*, 2004; Iemitsu *et al.*, 2006; Gavin *et al.*, 2015). Since it has been demonstrated that dynamic high intensity training enhances capillarization in human skeletal muscles (Jensen *et al.*, 2004), and because acute bouts of heavy intensity exercise elevate the concentrations of circulating PMVs in humans, we hypothesised that MVs produced during exercise would act on the vascular endothelium and stimulate angiogenesis. To test this hypothesis, we treated human endothelial cells seeded onto basement membrane matrix proteins with circulating MVs obtained from humans at rest and during heavy exercise. Exercise MVs increased the number of endothelial tubule-like structures in comparison to rMVs and their respective MV-free supernatants, suggesting that MVs produced during exercise may enhance the angiogenic activity of the endothelium during exercise. Because PMVs have been found to increase during exercise it seems plausible that this MV population would be involved with the angiogenic response. In agreement, MVs obtained from thrombin-stimulated platelets have been shown to display pro-angiogenic potential *in vitro* (Kim *et al.*, 2004; Brill *et al.*, 2005), and *in vivo* (Brill *et al.*, 2005), which may indicate that the PMV could be the MV population

responsible for bringing about the observed angiogenic effects in the present experiments.

By staining the proliferation-related protein Ki-67, Jensen *et al.* (2004) demonstrated that the increased skeletal muscle angiogenesis observed after training is in fact associated with migration and proliferation of endothelial cells forming new capillaries, and the results of the current study indicate that pro-angiogenic effects of MVs released during exercise may be supported by their stimulatory effects that induce endothelial migration and proliferation. It is worth noting that exercise increased the concentration of PMVs in the intravascular space and this might seem counterproductive since MV stimulation on the apical side of endothelial cells may stimulate migration into the vessel luminal space. However, under specific chemoattractant stimulation platelets may undergo transmigration through an activated endothelium (Kraemer *et al.*, 2010) and potentially deliver MVs beneath the basal surface of endothelial cells. Moreover, PMVs have been reported to adhere preferentially to the subendothelial extracellular matrix in comparison to endothelial cells and to serve as a binding site for platelets on the vessel wall (Merten *et al.*, 1999). The precise interplay between circulating MVs, endothelial migration stimulation, and angiogenesis is still far from understood, but one could speculate that PMVs might bind to points where the extracellular matrix is exposed and support vessel repair and angiogenesis. However, it is difficult to evaluate the relevance of this hypothesis during exercise without evidence of vascular damage.

The mechanisms responsible for exMVs induced morphogenic and mitogenic effects were not a focus of this study, yet a number of candidate mechanisms exist. One potential pathway through which MVs bring about their effects may be VEGF-related (Brill *et al.*, 2005; Benameur *et al.*, 2010). VEGF binds with high affinity to fms-like tyrosine kinase 1 and fetal liver kinase 1, with subsequent angiogenic and proliferative responses associated with the activation of several downstream effectors and second messengers, including protein kinase B and cyclic guanosine monophosphate related-pathways (Morbidelli *et al.*, 1996; Ziche *et al.*, 1997; Kitamura *et al.*, 2008). eNOS activation also seems to be required to mediate the proliferative and angiogenic effects of VEGF (Morbidelli *et al.*, 1996; Ziche *et al.*, 1997; Fukumura *et al.*, 2001). Human MVs have been shown to carry active forms of

eNOS (Horn *et al.*, 2013), and the *in vitro* pro-angiogenic effects of PMVs may be inhibited by VEGF receptor blockade (Brill *et al.*, 2005), suggesting a potential mechanism through which MVs increase endothelial motility, mitogenesis, and morphogenesis. It is worth also noting that depletion of lipids from PMVs also abolishes their angiogenic effects (Kim *et al.*, 2004), suggesting biologically active lipids as further mediators of MV functions.

7.5.1. Limitations

The results of the current study suggest a positive role for MVs, but also exhibit some limitations. Even though efforts were made to isolate circulating MVs, one cannot rule out that other agents might be mediating the observed effects. This, however, is still an intrinsic issue of this research field and the current study used an accepted approach applied by others to investigate the impact of circulating MVs in the vascular endothelium (Boulanger *et al.*, 2001; Vanwijk *et al.*, 2002). Moreover, the results with MV-free plasma provide further support that our findings arose from MV stimulation and not additional soluble molecules. One should also take into account that current methods for MV isolation do not enable the separation specific MV populations from human plasma without *ex vivo* agonist stimulation, and even though it seems logical that PMVs were the primary mediators of our findings, we cannot reject the hypothesis that other MV populations induced the observed responses. Furthermore, due to the dynamic nature involved in MV formation it remains unknown whether the influence of exMVs on endothelial cells resulted simply from their greater concentration in this condition, or due to distinct intrinsic characteristics of MVs produced during exercise (*e.g.* different cargo). Finally, it is essential to keep in mind that observations taken from static cell culture experiments are not necessarily transferable to other cell lines, and within complex whole organism models. Nevertheless, our results serve as a first step in this exciting new field of study in exercise sciences.

7.6. Conclusion

In conclusion, plasma MVs produced during exercise displayed a regenerative and pro-angiogenic potential in cultured endothelial cells when compared to circulating MVs from resting humans, which were supported by the stimulatory effects of exMVs on endothelial proliferation and migration, since these two processes are essential for angiogenesis and wound repair. Together, this set of data offers the first evidence of the biological role of exercise-derived MVs upon cells of the human vasculature, and provides evidence of a novel mechanism that may help us to uncover how exercise mediates vascular adaptations.

Chapter 8 - General Discussion and Conclusions

8.1. Introduction

The major aims of this thesis were to investigate the circulating PMV and EMV dynamics, the stimuli governing their formation during exercise, and to further explore the potential impact of circulating MVs upon endothelial cells *in vitro*. The human experiments described here explored the effect of large and small muscle mass exercise on arterial and venous concentrations of MVs and their appearance kinetics with exercise, focusing primarily on exercise-induced haemodynamic adjustments as the latent mechanism inducing PMV formation; whilst *in vitro* assays were used to examine proliferative, chemotactic and angiogenic potential of circulating MVs formed during exercise on human endothelial cells. More specifically, chapter 5 examined the time-course of appearance of venous PMV and EMV during two intensities of prolonged submaximal exercise and recovery, in order to explore the time-course of MV appearance in the circulation and further examine the potential relationships between vascular shear stress and additional biochemical agonists as mediators of intravascular MV release. Chapter 6 covered the a-v PMV and EMV responses to small and large muscle mass exercise, and the influence of vascular shear stress manipulations on local and systemic MV formation was explored with the aid of local thermal manipulations and single limb exercise. Finally, in chapter 7 the biological relevance of MVs was examined in cell culture to gain insights into the potential agonist effects of exercise-derived MVs on endothelial cell proliferation, migration, wound-healing, and angiogenesis.

The following chapter will centre on the interpretation of the main findings of this thesis from an integrative physiology perspective and in comparison to the literature, followed by a discussion of intrinsic methodological aspects of the studies. Finally, perspectives for future research in the area will be presented.

8.2. Main findings

8.2.1. Microvesicle dynamics

8.2.1.1. Platelet microvesicles during exercise

Our understanding of exercise as a physiological stress capable of stimulating MV blebbing, is very recent as most of the literature in this area has been published within the last decade. This means that several aspects related to MV dynamics in response to exercise and their physiological relevance are largely unexplored. Increases in plasma PMVs have been described in samples taken from the venous circulation far from exercising limbs (*e.g.* antecubital vein blood samples during leg cycling) during recovery from a variety of exercise protocols ranging from prolonged submaximal (Sossdorf *et al.*, 2010, 2011) to short duration very heavy or severe dynamic exercise (Chaar *et al.*, 2011; Maruyama *et al.*, 2012), but no study to date has explored the dynamics of PMVs during exercise. In chapter 5, we showed for the first time that [PMV] increase during exercise, and chapter 6 demonstrated that this elevated appearance of circulating PMVs occurs in both the arterial and venous circulations. During large muscle mass exercise, however, the a-v PMV difference was different from zero. This suggests a release of MVs by platelets that appear in the venous circulation, because the rise in [PMV] occurred in both the arterial and venous samples but it tended to be greater in the venous circulation draining exercising limbs. The mechanisms linked to this increased PMV release in the venous circulation are unknown, but it is possible that local factors stimulate the activation of calcium dependent (Fox *et al.*, 1991; Chang *et al.*, 1993; Dachary-Prigent *et al.*, 1995) and independent (Cauwenberghs *et al.*, 2006) pathways that lead to PMV formation when platelets are travelling through the microcirculation of active limbs during large muscle mass exercise.

In chapter 5, we demonstrated that PMV formation depends on the exercise stimulus, as PMVs were unaltered with moderate exercise (~35% PPO), but increased during heavy cycling (~60% of PPO). Previous studies investigating plasma PMVs applied exercise protocols varying from 45% to 100% of PPO, and consistently reported elevations in [PMV] after exercise (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Maruyama *et al.*, 2012). Understanding the effects

of exercise intensity was not the focus of chapter 6, but its results also provide some support for the suggestion that exercise intensity is a modulator of the PMV responses, since very heavy (80% PPO) large and small muscle mass exercise triggered an increase in PMVs. Another interesting observation from this thesis is that the increases in PMVs were similar amongst the 3 human experiments (Figure 8-1), despite the rather distinct exercise stimuli (*e.g.* from moderate to very heavy and short to prolonged duration exercise performed with either small or large muscle mass). This does not preclude exercise intensity as a factor involved in the stimulation of PMV formation, but rather indicates that there might be a threshold for platelet stimulation after which a plateau in [PMV] occur. After this theoretical point, additional mechanisms increasing MV clearance or preventing their release probably limits further appearance of PMVs in the circulation. Although it ought to be confirmed in future studies, this hypothetical "capping effect" in [PMV] may be a protective response to avoid the establishment of an excessive pro-coagulant environment induced by uncontrolled formation of MVs (Sossdorf *et al.*, 2010, 2011) which could dysregulate the body's haemostatic control.
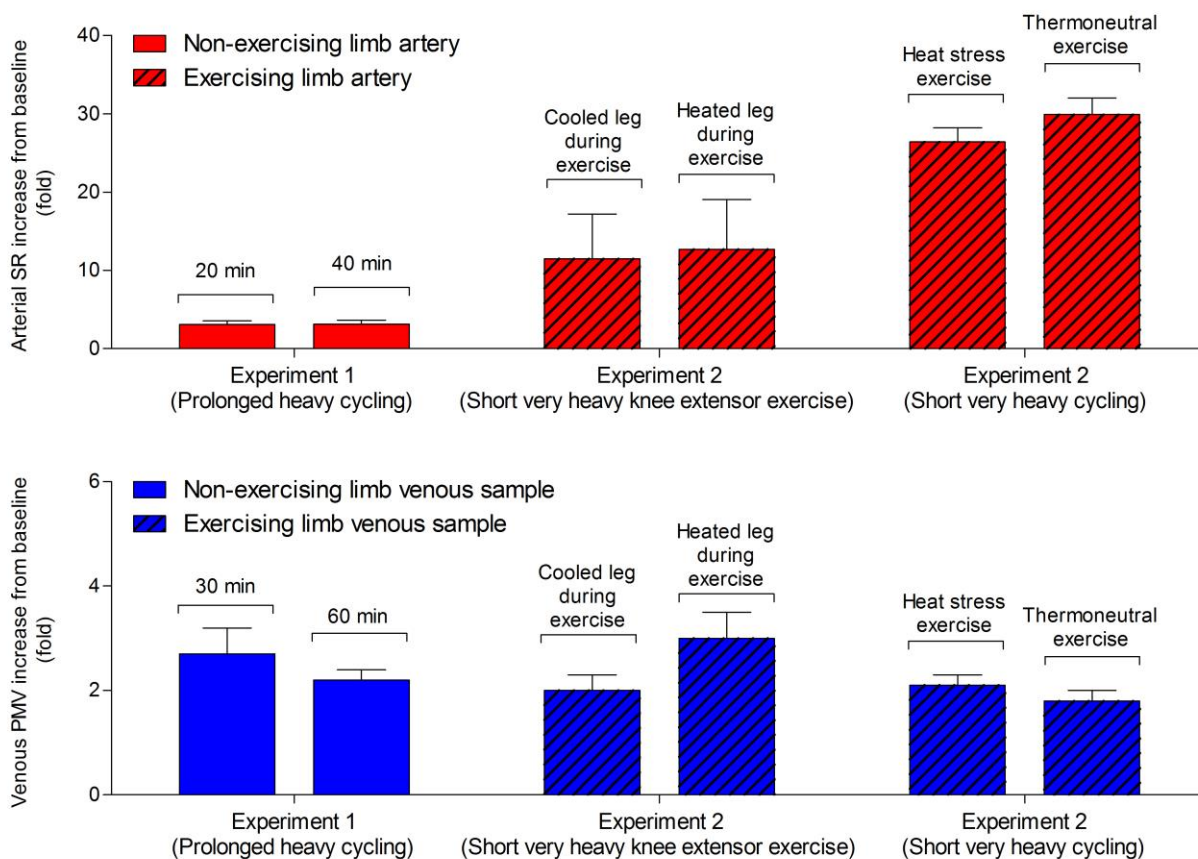
Figure 8-1. Baseline fold increase in arterial vascular shear rate (top) and venous plasma platelet microvesicle (PMV) concentrations (bottom) measured at the level of non-exercising limbs (study 1), and of exercising limbs (study 2) in the three human experiments of this thesis. During heavy and very heavy exercise, PMVs increased approximately 2 fold in the circulation of non-exercising and exercising limbs, whereas rather distinct SRs were observed in arteries feeding non-exercising and exercising limbs. Data are mean±SEM for 5 – 9 participants. Short very heavy cycling SRs are estimations, as described in chapter 6.

8.2.1.2. Platelet microvesicles during post-exercise recovery

Previous studies focused on the MV response shortly after exercise and during 2 h of recovery. Data in the literature allow us to speculate that the rise in circulating PMVs with large muscle mass exercise probably reaches its peak within 1 h of recovery (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Maruyama *et al.*, 2012). Our enhanced temporal resolution results from chapter 5 confirmed that platelets continue to release MVs in the early recovery period, which may persist for up to 1 h after heavy exercise. Indirect evidence for such post-exercise MV kinetics is also found in chapter 6, where circulating PMVs tended to be higher than baseline during the recovery from the first bout of very heavy exercise.

This MVs release might be supported by platelet activation during exercise, which may remain active during recovery when recruitment of new platelets may also occur (Davis *et al.*, 1990; Ikarugi *et al.*, 1999). From a practical perspective, these results demonstrate that the exposure of the human vasculature to augmented concentrations of PMVs is not limited to the exercise period, enabling these MVs to induce their intravascular effects for around 1 h after cessation of heavy exercise.

8.2.1.3. Endothelial microvesicles

Endothelial cells have been shown to produce MVs under several physiological conditions. Cultured endothelial cells release MVs following activation and apoptotic stimulation (Jimenez *et al.*, 2003), and augmented [EMV] have been linked to a wide spectrum of pre-clinical and clinical conditions ranging from obesity to coronary artery disease, and are thought to reflect vascular damage in these populations (Bernal-Mizrachi *et al.*, 2003; Esposito *et al.*, 2006; Horn *et al.*, 2013).

The appearance of circulating EMV with exercise has been investigated recently. The original work by Mobius-Winkler *et al.* (2009) described no changes in plasma EMVs with 4 h of moderate intensity cycling, with more recent work reporting conflicting results with either an increase (Sossdorf *et al.*, 2011; Kirk *et al.*, 2013; Lansford *et al.*, 2015), no change (Sossdorf *et al.*, 2010; Chaar *et al.*, 2011; Sossdorf *et al.*, 2011; Guiraud *et al.*, 2013; Ross *et al.*, 2014), and even reductions in circulating [EMV] (Wahl *et al.*, 2014) observed after exercise. It is currently difficult to determine which factors lead to these divergent findings in the literature, but the results of this thesis consistently demonstrate a lack of change in plasma [EMV] across a range of exercise intensities, exercise modalities and durations. The only condition in the present thesis in which [EMV] increased was a combination of heat stress and very heavy exercise, and remarkably these increases in concentration were completely explained by reductions in plasma volume. Changes in plasma volume during exercise have been overlooked by other researchers. For example, Sossdorf *et al.* (2011) reported a slight rise in participants' Hct accompanied by an increase in venous [EMV] in one of their experimental groups. It is possible that the ~25% elevation in EMVs in Sossdorf *et al.* (2011) work might have been partially

explained by reductions in plasma volume although it is difficult to estimate blood volume changes without Hb information. Nevertheless, it is likely that the modifications in [EMV] with exercise may at least in part be a consequence of simple haemoconcentration, and do not exclusively represent activation of the vascular endothelium with subsequent MV release.

8.2.2. Insights into platelet microvesicle formation in humans

The stimulation of *ex vivo* platelets with mechanical and biochemical factors have allowed scientists to identify a number of agonists that induce PMV formation and their respective downstream related pathways (Fox *et al.*, 1991; Dachary-Prigent *et al.*, 1995; Cauwenberghs *et al.*, 2006; Reininger *et al.*, 2006). These *in vitro* findings are extremely relevant from a mechanistic view point, but it is also important to appreciate that *ex vivo* results are not always transferable to *in vivo* situations, where a myriad of factors interact to maintain homeostasis. Hence, it is necessary to stress that a reductionist approach cannot explain the multifaceted processes involved in physiological responses since the activation of several complementary, often redundant, mechanisms can ensure an appropriate outcome (Joyner, 2013). In this thesis, therefore, we attempt to gain insight into a few potential mechanisms involved in MV formation during exercise with a particular emphasis on vascular shear forces, since they can activate vesiculation of platelets e*x vivo* (Miyazaki *et al.*, 1996; Reininger *et al.*, 2006) and they increase progressively throughout the vascular tree until arteriolar beds (Papaioannou *et al.*, 2006).

Accordingly, a novel aspect of this thesis was the investigation of potential stimuli related to the production of PMVs in exercising humans. Past studies have described the MV response after exercise protocols, but no effort has been made to understand the mechanisms explaining such responses. *In vivo* results from the three experiments of the thesis demonstrated that [PMV] display a weak to moderate correlation with vascular SR. Data from chapter 6, however, imply that vascular shear stress is not a key regulator of the localised production of PMVs, as their concentration increased simultaneously in the venous circulation of exercising and non-exercising limbs where SRs differed considerably. In addition, changes in [PMV]

during passive heating and/or exercise were relatively small in comparison to the marked differences in estimated leg vascular SRs induced by the different protocols (Figure 8-2). These results, however, do not rule out vascular shear stress as an *in vivo* agonist since platelets might be rapidly stimulated to produce MVs through local shear forces and then these platelets may quickly travel and release MVs systemically, even if they were activated in the exercising limb. Nevertheless, it seems that alternative systemic regulators are also involved in PMV formation during exercise. Noradrenaline, for example, is a platelet agonist that induces MV release *in vitro* (Tschuor *et al.*, 2008), and a positive correlation between circulating PMV and noradrenaline levels was reported in chapter 5. Additional intravascular platelet agonists present in the systemic circulation (that unfortunately could not be determined), such as thrombin (Brill *et al.*, 2005) and nucleotides (Yegutkin *et al.*, 2007), might also be involved with PMV formation, although the ability of the latter to promote PMV release still has to be confirmed.
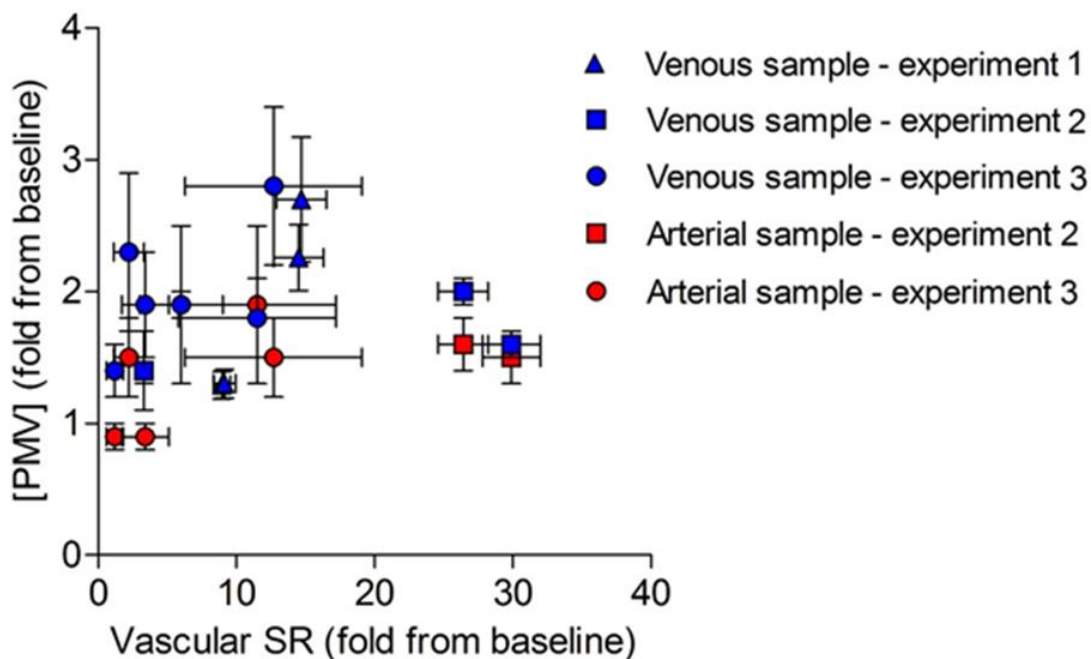
Figure 8-2. Plasma platelet microvesicle (PMV) and estimated or calculated common femoral artery shear rate (SR) fold change from baseline during passive heating, small and large muscle mass exercise in the three human experiments. Femoral artery SRs were predicted from blood flow estimations during experiment 1 (semi-recumbent cycling), and estimated from thermodilution blood flow measurements in experiment 2 (upright cycling), assuming baseline arterial diameters as a reference; vascular SRs were calculated directly from ultrasound measurements in experiment 3 (knee extensor exercise trial).

Hence, this thesis provides initial evidence for the understanding of mechanical and biochemical agents related to PMV formation during exercise. Although increased vascular shear stress might be involved to a degree with PMV formation, there is certainly not one single factor driving the systemic changes in [PMV] during exercise. Future experiments ought to expand these findings by using isolated shear stress manipulations with select agonists, and/or platelet vesiculation stimulation/blockade with exercise responsive biochemical agonists *in vivo*.

8.2.3. *Circulating microvesicles and endothelial cell interactions*

The physiological function of a variety of specific subpopulations of MVs has been intensively studied, which has changed our view of MVs from simple cell dust or by-products to biologically active cell biomarkers (Hargett & Bauer, 2013). Several studies have pointed to circulating MVs as putative regulators of vascular function *in situ* (Boulanger et al., 2001; Vanwijk et al., 2002), inducing a variety of stimulatory and inhibitory effects in cultured endothelial cells depending on the MV source and stimulus of formation (Brodsky *et al.*, 2004; Kim *et al.*, 2004; Brill *et al.*, 2005; Curtis *et al.*, 2009). This well-known MV – endothelial interaction led us to hypothesise that MVs circulating in the vasculature during exercise could be part of the intricate mechanisms through which exercise stimulates its beneficial vascular effects such as enhanced endothelial function and the formation of new capillaries.

As a follow up of chapters 5 and 6, where increased plasma concentrations of PMVs were consistently observed in healthy men performing short very heavy and prolonged heavy intensity exercise, in chapter 7 we provided the first evidence that physiological concentrations of circulating MVs are likely to be involved with endothelial responses to exercise, since cells treated with exMVs displayed enhanced proliferation and migratory capacities, which support a pro-angiogenic influence. The use of a MV-free internal control validated these findings by demonstrating that plasma obtained during exercise but depleted of MVs did not produce the same stimulatory effects. Intrinsic features related to circulating MV isolation obviously limit our capacity to clearly identify the contribution of specific MV subpopulations in the experimental assays. The [PMV], however, was greater in

exMV compared to rMV treatments, and Kim *et al.* (2004) demonstrated that PMVs promote a dose-dependent stimulation of endothelial proliferation and angiogenesis. This MV population has also been identified as an intravascular source of VEGF (Brill *et al.*, 2005), which suggests PMVs as a potential factor inducing angiogenic effects in cell culture observed in this thesis. Taken together, results from previous studies investigating the impact of MVs outside the context of exercise in addition to the present findings of this thesis suggest that exercise-derived MVs might be a novel candidate mechanism for vascular adaptation with exercise training. Whether these *in vitro* responses will transfer to the *in vivo* situation, however, remains to be determined in future experiments.

8.3. Significance of findings

Altogether, the human and cell culture experiments of this thesis represent an integrative approach that enhances our understanding of the complex interplay between acute haemodynamic changes and circulating factors that eventually may lead to enhanced endothelial adaptations with exercise training (Padilla *et al.*, 2011a). Based on the experimental chapters of this thesis and existing literature, a theoretical integration of blood MV responses to exercise and their physiological outcomes can be proposed.

In this hypothetical model, elevations in PMVs may be part of an integrative response to exercise that complement the effects of shear stress in inducing endothelial adaptations (Figure 8-3). As exercise intensity increases, both vascular shear stress and blood noradrenaline concentrations become elevated. These and additional factors, such as intravascular ADP, likely act together to activate platelets (Miyazaki *et al.*, 1996; Reininger *et al.*, 2006; Yegutkin *et al.*, 2007; Tschuor *et al.*, 2008), culminating in MV production after an agonistic threshold level is attained. The increased concentrations of MVs may play a role in the complex thrombotic-fibrinolytic control with exercise (Sossdorf *et al.*, 2010, 2011) whilst also being taken up by endothelial cells (Terrisse *et al.*, 2010; Dasgupta *et al.*, 2012), which in turn may stimulate endothelial repair and angiogenesis through direct MV – endothelial interactions. Although still speculative, increased blood noradrenaline concentrations

may negatively affect endothelial cells (Kaplon et al., 2011), and it might be possible that the increase in PMVs with exercise is a compensatory response to the increased blood catecholamine levels necessary for haemodynamic regulation within these conditions. A lack of change in plasma concentrations intuitively leads to the idea of no MV formation, but it is also possible that both the MV release and its uptake might be increased during moderate exercise. If that were the case, the intracellular concentration of MVs could be increased in endothelial cells, which may stimulate the vascular responses even if the net concentrations of MVs in the circulation remain unaltered. Whether or not the concentration of circulating MVs during moderate exercise actually reflects a matched increase in their formation and uptake ought to be determined. Overall, this hypothetical construct provides an integrative view of the physiological processes involved with exercise-induced MV formation and their potential impacts related to vascular adaptations. Future experiments will be required to confirm the validity and relevance of this hypothetical model.
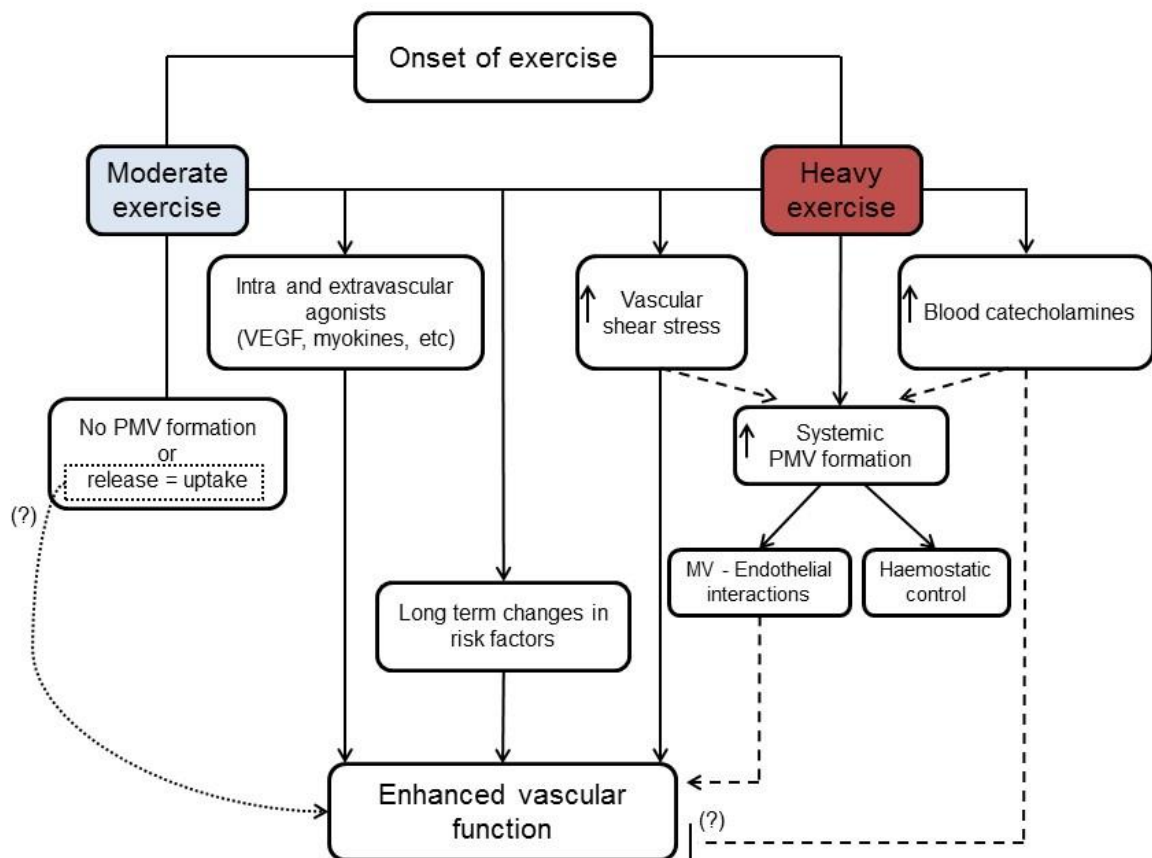
Figure 8-3. Theoretical model depicting stimuli for exercise-induced PMV formation and the potential biological relevance of circulating MVs in vascular function. As heavy or very heavy exercise progresses, vascular shear stress increases alongside blood catecholamine levels and several endothelial agonists. Vascular shear stress is likely to be the major stimulus for vascular adaptations (Tinken *et al.*, 2010; Birk *et al.*, 2012); whereas evidence suggests that elevations in blood noradrenaline concentrations have a depressor effect on endothelial function (Kaplon *et al.*, 2011). The interplay between these two factors potentially promotes PMV release during exercise, which may interact positively with endothelial cells as a compensatory mechanism to increased circulating catecholamines and add to shear stress stimulation and the contribution of chronic amelioration in cardiovascular risk factors. Circulating [PMV] does not increase during light exercise, which may reflect a lack of formation or alternatively a matching between increased MV shedding and clearance.

8.4. Methodological considerations

Although *in vitro* assays are performed under very strictly controlled experimental conditions, variations amongst individuals markedly increase variability and data dispersion with *in vivo* experiments. Likewise, the sample size in human experiments within this thesis (n = 9 for chapter 5, and n = 7 and 5 for MV results of chapter 6) may be considered small, which intrinsically increases the chances of

type II statistical error. Attaining so called "appropriate" levels of statistical power is a common issue in human and exercise physiology, with invasiveness and the difficulties of some procedures frequently limiting sample sizes, not to mention logistical and economic constraints. This was the case within the invasive experiments of chapter 6 where arteriovenous samples were obtained, whereas the time-consuming nature of experiments in chapter 5 restricted the number of volunteers willing to partake in prolonged experimental sessions (*i.e.* > 5 h involved in each main trial). To overcome potential issues related to sample size certain procedures were used, including a many-to-one comparison (Dunnett's test) instead of a general all possible pairwise-comparisons *post hoc,* to increase statistical power. However, the main dependent variables (*i.e.* MVs) frequently displayed changes in magnitude of ~100% or more during exercise, with effect sizes for PMVs varying from 1.4 to 1.5 during heavy exercise in chapter 5 and from 1 to 1.6 in chapter 6, which are far from trivial. Thus, even though larger sample sizes would be beneficial from a statistical point of view, it is more reasonable to consider that the results of this thesis fall within the standards of human physiology experiments of this nature.

In this thesis circulating MVs were determined using a state-of-art imaging flow cytometer (ImageStream^X® Mark II, Amnis Corporation, USA), rather than a traditional cytometer. Imaging flow cytometry is a relatively new technique that allows not only the acquisition of positive events, but the identification and image analysis of each individual event. According to the "iceberg hypothesis", only a small portion of MVs fall within the detection limits of traditional laser flow cytometers (Harrison & Gardiner, 2012). The 60x objective magnification in our imaging flow cytometer provided us with enhanced resolution for size discrimination compared to standard cytometers (Harrison & Gardiner, 2012; Headland *et al.*, 2014), which in all likelihood allowed us to perform MV quantification with a much improved limit of detection compared to past studies. Hence, due to the improved resolution and meticulous titration of antibodies to optimize MV analyses, findings resulting from this thesis should be considered a step forward in our understanding of MV dynamics with exercise, even though their absolute values might not compare directly to previous research in the field using traditional flow cytometric techniques.

Finally, an important characteristic of circulating MVs relates to their phenotype in response to specific agonists. PMVs can be identified by a variety of surface antigens that are believed to be expressed independent of the platelet agonist. Endothelial cells, however, are known to secrete MVs with distinct markers depending on their intracellular state (Jimenez *et al.*, 2003). It is important, then, to draw attention to our CD62E$^+$ events, which are thought to reflect EMVs released by activated endothelial cells; however, we cannot rule out the presence of other EMV populations arising from other endothelial stimuli, such as those released by apoptotic endothelia.

8.5. Future perspective

The present thesis explored a number of interactions between exercise, intravascular MVs, and the vascular endothelium. Although the present findings expand our current knowledge, they certainly do not cover all aspects of this relatively new research field. Hence, directions for future investigations will be presented below.

Results from this thesis demonstrate the importance of exercise intensity in PMV formation. However, the precise intensity at which significant elevations in MV appearance occurs was not determined. Since circulating MVs formed during exercise were initially seen as novel postulants of positive endothelial responses in this thesis, the identification of a minimal workrate necessary to bring about increases in blood MVs could be of practical significance for future training related research in the applied setting. This could be accomplished using maximal incremental test protocols with blood sampling performed at each exercise step. Although, this could have been completed in chapter 6 of this thesis, logistical restrictions limited the number of blood samples available for MV quantification, and this topic will have to be address in future experiments.

The acute influence of circulating MVs formed during exercise on the proliferation and morphogenesis of cultured endothelial cells were also explored in this thesis, but their importance *in vivo* and relevance in relation to long term

vascular adaptations with exercise remain to be determined in experiments where exercise-derived MVs are applied in *in vivo* models, and in which blockade of the increase in circulating MVs during exercise is performed. Blunting the release of PMVs during acute exercise might be possible in experiments using high doses of platelet antagonists, such as acetylsalicylic acid, and could provide further mechanistic insight about PMV formation during exercise and their subsequent effects on endothelial cells. Longitudinal experiments with chronic inhibition of MV formation might be difficult to perform in humans, but the investigation of long term vascular responses to exercise amongst Scott syndrome patients (a rare congenital bleeding disorder due to defects in platelet plasma membrane remodelling and MV release) may provide the first insight into the importance of acute changes in MVs on chronic endothelial adaptations. This would have clinical relevance amongst Scott syndrome and other patient populations and may ultimately lead to the development of training strategies to optimise the prevention of vascular dysfunction.

Moreover, future research involving the *in vitro* blockade of intracellular pathways would help identify mechanisms through which exercise-derived MVs promote their positive effects on endothelial cells. This could expand our knowledge about vascular physiology and might help identify novel targets for the promotion of vascular health.

8.6. Hypotheses

8.6.1. Study 1

1) Plasma [PMV] increase during and after exercise, according to relative exercise intensity **(accepted)**

2) PMVs return to baseline values in the late recovery period **(accepted)**

   Changes in [PMV] during exercise correlate with:

   3) Vascular SR **(accepted)**

   4) Plasma noradrenaline concentrations **(accepted)**

5) Plasma IL-6 concentrations **(rejected)**

6) Plasma [EMV] remain stable with submaximal exercise **(accepted)**

8.6.2. Study 2

1) Circulating [PMV] increase during passive whole-body heat stress and exercise **(accepted)**

2) Localised shear stress manipulations coincide with local changes in venous PMVs during single limb heat stress and exercise **(rejected)**

3) [EMV] remain stable during passive heat stress but increase with very heavy large muscle mass exercise **(accepted)**

8.6.3. Study 3

Compared to other conditions, circulating MVs obtained during exercise stimulate greater endothelial cell:

1) Proliferation **(accepted)**

2) Migration **(accepted)**

3) Healing from a scratch-wound **(accepted)**

4) *In vitro* angiogenesis **(accepted)**

8.7. Summary of findings

Throughout prolonged leg cycling and recovery [EMV] measured at a vein from the antecubital region remained unaltered, whereas PMVs increased and plateaued within 30 min of heavy, but not moderate, exercise. Changes in [PMV] during exercise were correlated with increases in peripheral vascular SR and plasma

noradrenaline concentrations. Elevated levels of PMVs were also observed 1 h after heavy cycling and returned to baseline in late recovery, demonstrating an early post-exercise effect on PMV dynamics.

Moderate, but not mild, passive heat stress augmented arterial [PMV], which remained elevated during short duration very heavy exercise. Venous concentration of PMVs tended to be elevated with moderate passive heat stress, and markedly increased during both large and small muscle mass exercise, leading to a tendency of PMV release in the venous circulation of active limbs during large muscle mass exercise. The concentration of EMVs, on the other hand, increased only in the venous circulation and during large muscle mass exercise under heat stress conditions, most likely reflecting haemoconcentration. Isolated manipulations of vascular SR revealed little association between local PMV production and vascular shear stress, indicating a systemic effect of exercise on PMV formation.

Lastly, circulating MVs isolated from humans during heavy intensity exercise enhance the proliferative and migratory capacity of cultured human endothelial cells compared to those isolated from resting humans. Moreover, parameters related to *in vitro* angiogenesis were also increased under these conditions, with minor effects of MV-free plasma controls.

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Appendix I – Ethical Approval

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Eurico Wilhelm Neto
PhD Researcher (Sport Sciences)
School of Sport and Education
Brunel University

13th March 2014

Dear Eurico

RE39-13 Time-course characterisation of circulating microparticles with exercise

I am writing to confirm the Research Ethics Committee of the School of Sport and Education received your application connected to the above mentioned research study. Your application has been independently reviewed to ensure it complies with the University/School Research Ethics requirements and guidelines.

The Chair, acting under delegated authority, is satisfied with the decision reached by the independent reviewers and is pleased to confirm there is no objection on ethical grounds to grant ethics approval to the proposed study.

Any changes to the protocol contained within your application and any unforeseen ethical issues which arise during the conduct of your study must be notified to the Research Ethics Committee for review.

On behalf of the Research Ethics Committee for the School of Sport and Education, I wish you every success with your study.

Yours sincerely

PP Dr Richard J Godfrey
Chair of Research Ethics Committee
School Of Sport and Education

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2011

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Brunel
UNIVERSITY
L O N D O N

Mr Steven Trangmar
PhD (Sport Sciences) Student
School of Sport and Education
Brunel University

6th September 2013

Dear Steve

RE54-12 The effect of heat stress on muscle, brain and systemic haemodynamics during incremental cycling exercise: partitioning the role of skin and internal temperature

I am writing to confirm the Research Ethics Committee of the School of Sport and Education received your application connected to the above mentioned research study. Your application has been independently reviewed to ensure it complies with the University/School Research Ethics requirements and guidelines.

The Chair, acting under delegated authority, is satisfied with the decision reached by the independent reviewers and is pleased to confirm there is no objection on ethical grounds to grant ethics approval to the proposed study.

Any changes to the protocol contained within your application and any unforeseen ethical issues which arise during the conduct of your study must be notified to the Research Ethics Committee for review.

On behalf of the Research Ethics Committee for the School of Sport and Education, I wish you every success with your study.

Yours sincerely

Dr Richard J Godfrey
Chair of Research Ethics Committee
School Of Sport and Education

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Mr Scott Chiesa
4 Belsize Avenue
Palmers Green
London
N13 4TJ

15 November 2011

Dear Scott

RE04-11 - The role of local tissue temperature on limb muscle blood flow

I am writing to confirm the Research Ethics Committee of the School of Sport and Education received your application connected to the above mentioned research study. Your application has been independently reviewed to ensure it complies with the University/School Research Ethics requirements and guidelines.

The Chair, acting under delegated authority, is satisfied with the decision reached by the independent reviewers and is pleased to confirm there is no objection on ethical grounds to the proposed study.

Any changes to the protocol contained within your application and any unforeseen ethical issues which arise during the conduct of your study must be notified to the Research Ethics Committee.

On behalf of the Research Ethics Committee for the School of Sport and Education, I wish you every success with your study.

Yours sincerely

Dr Gary Armstrong
Chair of Research Ethics Committee
School Of Sport and Education

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Appendix II - Informed Consent

CONSENT FORM

Study title

Participant's name & signature:_____Date:

Investigator's name & signature:_____Date:

The participant should complete the whole of this sheet himself		
	Please tick the appropriate box	
	YES	NO
Have you read the Information for Research Participant?	☐	☐
Have you had an opportunity to ask questions and discuss this study?	☐	☐
Have you received satisfactory answers to all your questions?	☐	☐
Who have you spoken to?		
Do you understand that you will not be referred to by name in any report concerning the study?	☐	☐
Do you agree for your blood samples to be analysed and used in future studies that may not be detailed in the information provided?	☐	☐
Do you understand that you are free to withdraw from the study:		
- **at any time**	☐	☐
- **without having to give a reason for withdrawing?**	☐	☐
- **without affecting your future care**	☐	☐
Do you agree to your GP being informed if necessary?	☐	☐
Do you agree to take part in this study?	☐	☐
Signature of Research Participant:	**Date:**	
Name in capitals:		
██		
Witness statement *'I am satisfied that the above-named has given informed consent.'*		
Signature of Witness:	**Date:**	
Name in capitals:		

Appendix III – Health Questionnaire

PRE-PARTICIPATION HEALTH CHECK QUESTIONNAIRE

Health and safety within this investigation is of paramount importance. For this reason we need to be aware of your current health status before you begin any testing procedures. The questions below are designed to identify whether you are able to participate now or should obtain medical advice before undertaking this investigation, Whilst every care will be given to the best of the investigators ability, an individual must know his/her limitations.

Subject name………………………………………………………………………………

Date of birth…...………………………………………………………...………….

Emergency contact name and number:………………………………………....

Please answer the following questions:

	NO	YES
Has your doctor ever diagnosed a heart condition or recommend only medically supervised exercise?	☐	☐
Do you suffer from chest pains, heart palpitations or tightness of the chest?	☐	☐
Do you have known high blood pressure? If yes, please give details (*i.e.* medication)	☐	☐
Do you have low blood pressure or often feel faint or have dizzy spells?	☐	☐
Do you have known hypercholesteremia?	☐	☐
Have you ever had any bone or joint problems, which could be aggravated by physical activity?	☐	☐
Do you suffer from diabetes? If yes, are you insulin dependent?	☐	☐
Do you suffer from any lung/chest problem, *i.e.* Asthma, bronchitis, emphysema?	☐	☐
Do you suffer from epilepsy? If yes, when was the last incident?	☐	☐
Are you taking any medication?	☐	☐
Have you had any injuries in the past? *e.g.* back problems or muscle, tendon or ligament strains, etc…	☐	☐
Are you currently enrolled in any other studies?	☐	☐
I have already participated in a recent blood donation program	☐	☐
Are you a smoker?	☐	☐
Do you exercise on a regular basis (at least 60 min a week)?	☐	☐
Do you have known anaemia?	☐	☐

Describe your exercise routines (mode, frequency, intensity/speed, race times):

If you feel at all unwell because of a temporary illness such as a cold or fever please inform the investigator. Please note if your health status changes so that you would subsequently answer YES to any of the above questions, please notify the investigator immediately.

I have read and fully understand this questionnaire. I confirm that to the best of my knowledge, the answers are correct and accurate. I know of no reasons why I should not participate in physical activity and this investigation and I understand I will be taking part at my own risk.

Appendix IV – List of Cell Culture Supplies

Phosphate buffered saline (CAT# P4417-100TAB), medium 199 (M199 - Hyclone; CAT# SH30024.01), modified medium 199 (phenol red, l-glutamine, and sodium hydrogen carbonate-free; CAT# M3769), dimethyl sulfoxide (DMSO; CAT# 472301), trypsin-EDTA (CAT# 59418C-100ml), sodium citrate, thiazol blue tetrazolium bromide (MTT; CAT# M5655), gelatine solution (2%; CAT# G1393-100ML) were from Sigma-Aldrich (USA). Heparin sodium (CAT# 411210010), l-glutamine (CAT# AC119951000), and azur eosin methylene-blue (Giemsa stain solution; CAT# AC295591000) were from Acros Organics (by Fisher Scientific, UK). Sodium hydrogen carbonate (CAT# S/4200/60), HEPES (CAT# BP299-100), and VEGF (CAT# RP-75746) were from Fisher Scientific (UK). Trypan blue (CAT# SV30084.01), and penicillin-streptomycin (CAT# SV30010) solutions were from Thermo Fisher Scientific (UK). Foetal bovine serum (FBS – CAT# 10270-106), and Geltrex® (CAT# A1413202) were from Life Technologies (UK). Fc receptor blocking solution (Human Trustain FcX; CAT# 422302), PE anti-human CD62E (CAT#322606) and PE/Cy5 anti-human CD41 antibodies (CAT#303708) were from BioLegend (USA). Endothelial cell growth supplement (CAT# 4110-5004) was from Bio-Rad (UK). Pipette tips for the scratch wound-healing were from Fisherbrand, UK (1-200 µl, CAT# 10283882); and combitips used for coating plates for angiogenesis assay were from Eppendorf (0.5 ml, CAT# 12674587, Eppendorf, Germany).

Appendix V – Publication

EXERCISE INTENSITY MODULATES THE APPEARANCE OF CIRCULATING MICROVESICLES WITH PRO-ANGIOGENIC POTENTIAL UPON ENDOTHELIAL CELLS

Eurico Nestor Wilhelm, José González-Alonso, Christopher Parris, Mark Rakobowchuk

Appendix VI – Conference Abstract

CIRCULATING MICROVESICLE DYNAMICS WITH EXERCISE AND THEIR IMPACT ON HUMAN VASCULAR CELL FUNCTION

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Oral presentation at ECSS 2016, Vienna, Austria

Introduction: Microvesicle release into the bloodstream may be a signalling mechanism controlling the function of vascular cells, but the impact typical endurance exercise sessions on circulating concentrations, and the role played by these blood constituents on surrounding endothelial cells remains unknown. Here, we tested the hypothesis that exercise intensity would affect the time-course of platelet-derived (PMV) and endothelial-derived (EMV) microvesicle appearance in the circulation through changes in vascular shear stress and biochemical agonists; and that microvesicles obtained during exercise would enhance angiogenic and reparative potential of endothelial cells. **Materials and methods:** Nine young health men (25± 4 years, 1.79±0.09 m, 80.5±12.0 kg) had venous blood samples taken to measure plasma microvesicle, interleukin-6 (IL-6) and noradrenaline (NA) concentrations prior, during, and throughout the recovery period after 1 h of moderate (MI) (36±5% of VO_2max) or heavy (HI) (62±4% VO_2max) intensity semi-recumbent cycling and during a time matched control trial. Local haemodynamics were determined by ultrasonography. *In vitro* experiments were performed by incubating resting and exercise-derived microvesicles in cell culture to examine their effects on morphogenesis, proliferation and migratory capacity of human umbilical vein endothelial cells. **Results:** Circulating PMVs only increased from baseline with HI exercise (from $21.2\pm3.4^{.}10^3$ PMV$^{.}\mu l^{-1}$ to $55.8\pm24.8^{.}10^3$ and $48.0\pm16.6^{.}10^3$ PMV$^{.}\mu l^{-1}$ at 30 and 60 min, respectively; P<0.05), returning to baseline early in post-exercise recovery (P>0.05), whereas EMVs were unchanged (P>0.05). Vascular shear rate at the brachial artery (non-exercising limb) increased more during HI (3.1±1.5 fold) compared to MI (1.9±0.4 fold) exercise (P<0.05), and changes in PMV concentrations were related to limb shear rate (r^2=0.43, P<0.05), but not to IL-6 or NA (P<0.05). Incubation of microvesicles obtained during exercise with endothelial cells enhanced their proliferation, migration, healing of scratch wound (all P<0.05), and angiogenesis compared to microvesicles obtained at rest and microvesicle free plasma control. **Discussion:** These results demonstrate that circulating PMVs

increase during heavy exercise and that shear stress is a potential candidate mechanism triggering this PMV response. The increased microvesicle pool seemed to have agonist effects on human endothelial cells by stimulating angiogenesis and repair, and may be considered a novel mechanism through which exercise mediates vascular healing and adaptation.