Vascular health and repair in women across the lifespan: effect of exercise

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The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Introduction: Cardiovascular disease (CVD) is the main global cause of mortality with a death rate higher in females than males. Exercise training has the potential to reduce CVD risk but the optimal exercise type has not been determined. Therefore, the effects of two different types of exercise (interval and continuous) on markers related to CVD risk were assessed in women across the lifespan.

Methods: The effect of exercise on vascular health was studied in three different populations of women; young premenopausal, middle-aged overweight/obese, and postmenopausal women. In chapters 4 and 6, 12 healthy and 20 overweight/obese women, respectively, completed either an interval or continuous exercise training programme. In chapter 5, 15 postmenopausal women performed a 30 min moderate-intensity continuous and interval exercise bout, with 9 participants completing a further interval exercise session at a heavy-intensity. Endothelial function, arterial stiffness, circulating angiogenic cell (CAC) number and function, and cardio-respiratory fitness were assessed in these chapters at pre and post-exercise.

Results: Arterial stiffness was unaltered following exercise in all chapters. Cardio-respiratory fitness was increased following both interval and continuous exercise training. Brachial artery flow-mediated dilation (FMD) was increased following interval exercise training in young women, unaltered acutely in postmenopausal women, and decreased following interval exercise training in overweight/obese women. CAC number was increased following both types of exercise in young women, but in overweight/obese women, CAC number was only increased after interval exercise training, and was unaltered acutely in

postmenopausal women. Markers of CAC function were unaltered following exercise training in healthy young women, but CAC colony-forming units increased acutely following moderate and heavy-intensity interval exercise in postmenopausal women, and CAC adhesion increased following interval exercise training in overweight/obese women. However, continuous exercise (acutely and chronically) did not change endothelial function or CAC function in any study.

Discussion: Interval exercise modified more markers of vascular health than continuous exercise. The mechanisms behind this discrepancy might be related to potential differences in the arterial shear stress profiles experienced during the exercise. Future studies are required to explore this theory. Exercise-mediated changes in many variables depended on the baseline health of participants and therefore, highlights that exercise effects are heterogeneous.

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Chapter 1 Introduction

Cardiovascular disease (CVD) is the main cause of global mortality, and in Europe is responsible for 54% of female and 43% of male deaths (Allender *et al.*, 2008b). Despite the great burden of CVD in both sexes, women are at a lower risk of developing CVD across the lifespan compared to men, due to the atheroprotective effects of oestrogen. The incidence rates for CVD in women are at a 10 year lag behind men until the menopausal transition, where the risk of CVD rises in females but attains a plateau in men [Figure 1.1; Lerner & Kannel (1986)].

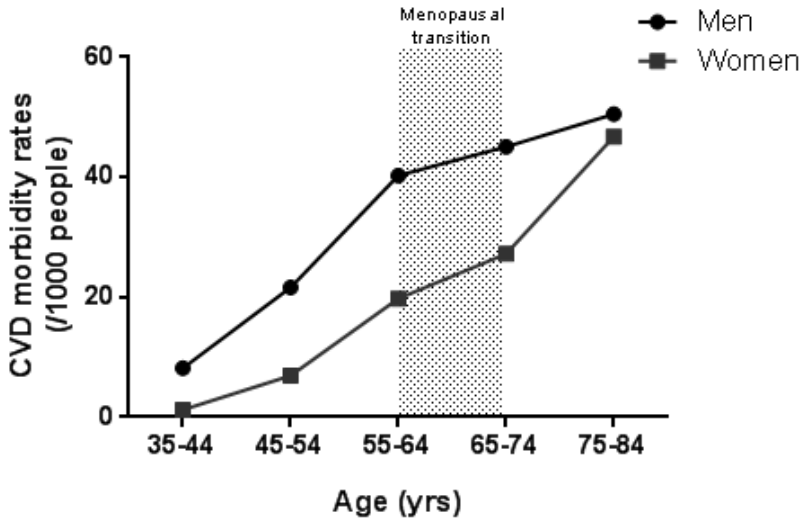

Figure 1.1. Incidence of cardiovascular disease (CVD) across the lifespan by gender: results of the Framingham study. Males have higher rates of CVD than women until older age, where this discrepancy is narrowed due to the loss of oestrogen at the menopause in women. Around the menopausal transition the risk of CVD in women increases while at this age in men, the risk plateaus. (Lerner & Kannel, 1986).

Although women have lower CVD morbidity rates, the CVD mortality rates per year are greater in women than men (Allender *et al.*, 2008a; Vaccarino *et al.*, 2009; Roger *et al.*, 2011). Moreover, the consequences are more severe, as 43% of women will die within 5 years of experiencing a first myocardial

infarction (MI) compared to 33% of men (Lloyd-Jones *et al.*, 2009). Furthermore, a greater percentage of women than men who die from a sudden cardiac death will not have previously been diagnosed with heart disease, or experienced symptoms (Lloyd-Jones *et al.*, 2009; Bertoia *et al.*, 2012). These high rates may be caused by a lack of awareness of CVD symptoms in women (Maas & Appelman, 2010), a difference in the presentation of CVD symptoms between gender (Anand *et al.*, 2005; Vaccarino *et al.*, 2009), and/or less aggressive treatment. Indeed, in 12,562 patients experiencing acute coronary syndrome, a higher percentage of men than women underwent coronary angiography, angioplasty, coronary artery bypass graft surgery and were given β-blockers, whilst a higher percentage of women were re-hospitalised with angina and had a greater number of diseased vessels (Anand *et al.*, 2005). Furthermore, angina is the first main symptom of CVD in women compared to an MI in men, and women are more likely to experience a “silent” MI than men (Lerner & Kannel, 1986). Despite the great burden of CVD and the difference in disease symptoms, treatment and awareness in women, fewer clinical trials and intervention studies have been conducted specifically in female populations (Wenger, 2012). Thus, this thesis will focus specifically on females across the lifespan, with and without CVD risk factors.

The underlying pathophysiology of CVD begins with atherosclerosis, a condition relating to the inflammation of the blood vessels, which is initiated by endothelial dysfunction (Ross, 1999). This condition is characterised by an increase in vascular damage that outweighs vascular repair and protective mechanisms, thereby creating an imbalance in vascular homeostasis. Arterial stiffening and a reduction in circulating angiogenic cell (CAC) number and function augment this process (the mechanisms of which are reviewed in chapter 2). Thus, improving

vascular homeostasis by lessening endothelial dysfunction and improving arterial compliance and CAC number and function, should be the first target of therapeutic and lifestyle interventions. Exercise has been identified as a non-pharmacological and cost-effective intervention for improving these factors (reviewed in chapter 2). Indeed, physical inactivity is the 4th main cause of global deaths caused by non-communicable diseases (World Health Organisation, 2009). Moreover, in women with CVD or presenting with CVD risk factors, regular exercise is one of the first recommendations for improving vascular health (Mosca *et al.*, 2011). However, fewer women than men participate in exercise, with only 4% achieving the recommended government guidelines of 30 min of moderate intensity exercise on 5 days per week (British Heart Foundation, 2012). These guidelines are vague and not population or gender specific, despite differences in CVD presentation and mortality between males and females, as discussed. Furthermore, the type and intensity of exercise that will yield the greatest improvements to vascular health in different populations of women (i.e. premenopausal, obese, and postmenopausal) is unknown. Recent evidence suggests that interval exercise involving variable work-rates, might be more enjoyable and superior for increasing endothelial function and arterial stiffness, than the traditional recommended method of continuous type exercise (Wisloff *et al.*, 2007; Rakobowchuk *et al.*, 2008; Tjønna *et al.*, 2008; Ciolac *et al.*, 2010; Guimaraes *et al.*, 2010). However, this comparison has not been studied specifically in different populations of women and moreover, the effects of interval and continuous exercise on CACs has not been investigated. Therefore, the main purpose of the thesis was to compare the effects of interval and continuous type exercise on markers of vascular health in different populations of women.

Chapter 2 Literature Review

2.1 The endothelium in health and disease

2.1.1 Overview

The endothelium is a single monolayer of cells that line the inner blood vessel walls and has a critical role in maintaining vascular homeostasis due to its direct contact with circulating blood. Healthy endothelial cells respond to physical and chemical stimuli in the blood by secreting a variety of growth factors, coagulants, vasodilators and vasoconstrictors that regulate vasomotor tone and act in combination to prevent inflammation and subsequent atherosclerosis (Rubanyi, 1993; Griendling *et al.*, 2000). Conversely, a dysfunctional endothelial cell switches to a pro-atherogenic phenotype through secretion of cytokines, inflammatory molecules and expression of adhesion molecules, which increases endothelial permeability, oxidative stress and leukocyte adhesion (Marti *et al.*, 2012). Thus, the measurement of endothelial function *in vivo* can be used as a prognostic indicator of CVD. Indeed, an impaired vasodilation in response to acetylcholine or shear stress in coronary and peripheral vessels can predict future cardiac events in CVD patients and indicate the severity of CVD (Neunteufl *et al.*, 1997; Cai & Harrison, 2000; Neunteufl *et al.*, 2000; Halcox *et al.*, 2002; Landmesser *et al.*, 2004; Green *et al.*, 2011). One of the most influential atheroprotective molecules produced by the endothelium is nitric oxide (NO).

2.1.2 The atheroprotective properties of nitric oxide

NO was first identified as possessing anti-atherogenic properties following its discovery as a potent vasodilator. Furchgott and Zawadzki in 1980 found that the endothelium was responsible for vasodilation through the secretion of an

endothelial-derived relaxing factor (EDRF) after the observation that denuded rabbit aortas did not dilate in response to acetylcholine. This EDRF was later identified as NO (Palmer *et al.*, 1987). Endothelial NO release also mediates other atheroprotective processes (Figure 2.1) including the inhibition of leukocyte adhesion and migration (Kubes *et al.*, 1991), vascular smooth muscle cell proliferation [VSMC; Rudic *et al*., (1998)], and platelet aggregation (Radomski *et al.*, 1990). Thus, the bioavailability of NO is key for maintaining vascular homeostasis.

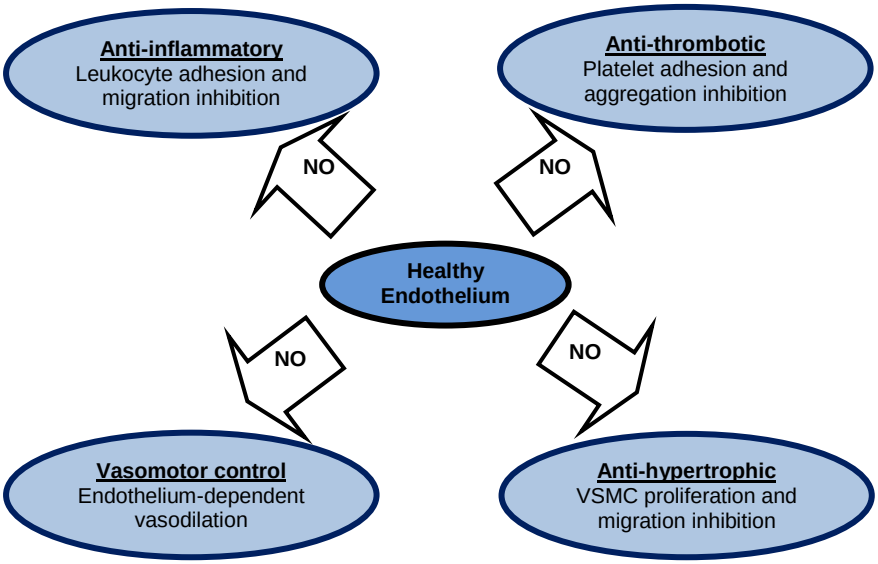

Figure 2.1. The atheroprotective properties of nitric oxide (NO). NO is released from endothelial cells and is a potent vasodilator and inhibits endothelial leukocyte adhesion and migration, platelet adhesion and aggregation and vascular smooth muscle cell (VSMC) proliferation from the tunica media. Modified from Landemesser *et al*., (2004).

2.1.3 Activation of eNOS and nitric oxide synthesis

The generation of NO in endothelial cells occurs through the conversion of L-arginine to L-citrulline by the enzyme endothelial nitric oxide synthase [eNOS; Palmer & Moncada, (1989)]. Upon activation of eNOS, electrons are transferred from nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) to flavin adenine dinucleotide (FAD) and then flavin mononucleotide (FMN) in the reductase domain, and passed to tetrahydrobiopterin (BH_4) and the haem iron

in the oxygenase domain, resulting in binding of oxygen and the oxidation of L-arginine to NO and L-citrulline [Figure 2.2; Alderton *et al*., (2001)]. Phosphorylation of eNOS at its serine site Ser^{1177} is a main activation pathway, which is stimulated by various factors such as vascular endothelial growth factor [VEGF; Michell et al., (1999)], oestrogen (Haynes *et al.*, 2000) and shear stress (Dimmeler *et al.*, 1999). Depending on the type of stimuli, intracellular pathways initiating eNOS phosphorylation can be calcium (Ca^{2+})-dependent or Ca^{2+}-independent. AMP-activated protein kinase (AMPK) phosphorylates Ser^{1177} and activates eNOS only when Ca^{2+} and calmodulin are present (Chen *et al.*, 1999). However, shear stress activates the phosphatidylinositol-3 Kinase (PI3K)/Akt pathway (Figure 2.2) independently of Ca^{2+}/calmodulin levels (Dimmeler *et al.*, 1999). Following eNOS activation, NO is synthesised and diffuses into the surrounding tissue where it exerts its atheroprotective effects.

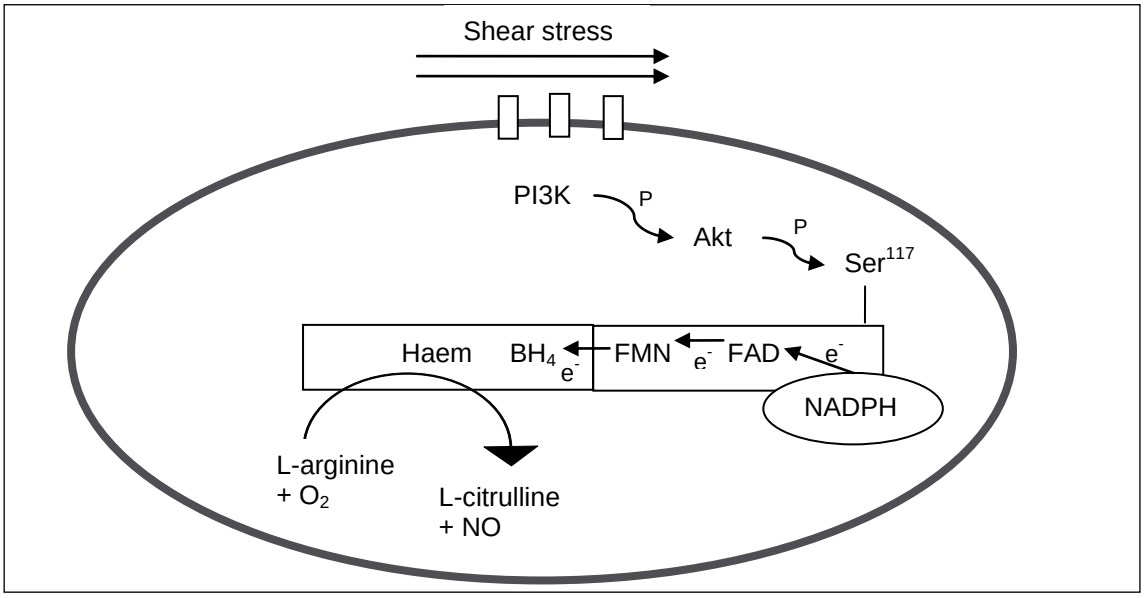

Figure 2.2. Activation of eNOS via the PI3K/Akt pathway. Shear stress is detected by mechanotransducers on endothelial cells leading to the activation of PI3k which phosphorylates Akt. Akt phosphorylates eNOS at its serine 1177 site (Ser^{1177}) allowing electron (e^-) transfer from NADPH to FAD to FMN in the reductase domain, and through to tetrahydrobiopterin (BH_4) and the haem iron in the oxygenase domain, resulting in the oxidation of L-arginine to L-citrulline and nitric oxide (NO). Adapted from Alderton *et al*., (2001).

2.1.4 Nitric oxide and vasodilation

As a potent vasodilator, NO induces the relaxation of vascular smooth muscle cells in the tunica media (middle layer of an artery). Upon synthesis of NO from the endothelial cells, NO diffuses into the surrounding vascular smooth muscle cells and activates soluble guanylate cyclise (sGC), which catalyses the dephosphorylation of guanosine triphosphate (GTP) to cyclic guanosine monophosphate [cGMP; Waldman & Murad, (1988)]. Subsequently, cGMP reduces the intracellular concentrations of Ca^{2+} and activates cGMP kinase, which activates myosin light-chain phosphatase resulting in VSMC relaxation by dephosphorylating myosin light-chains (Waldman & Murad, 1988). NO mediated VSMC relaxation can also occur independently of cGMP through the activation of potassium (K^{+}) channels (Bolotina *et al.*, 1994) which causes cell hyperpolarisation, forcing Ca^{2+} out of the cell enabling relaxation (Tare *et al.*, 1990). The magnitude of vasodilation in response to acetylcholine (Halcox *et al.*, 2002) or shear stress [flow-mediated dilation; FMD (Celermajer *et al.*, 1992)] can be measured using ultrasound imaging and is therefore used as an indirect measure of NO bioavailability and endothelial function (refer to the general methods, chapter 3, section 3.5 for details on the FMD technique). A reduction in NO bioavailability is one of the main mechanisms of endothelial dysfunction in many disease states.

2.1.5 Mechanisms of endothelial dysfunction

Endothelial dysfunction can be defined as an activation of endothelial cells towards an atherogenic state, which is characterised by a secretary imbalance between one or all of the following factors: vasodilators and vasoconstrictors, anti and pro-coagulants, growth-promoting and growth inhibiting factors and anti and pro-inflammatory molecules (Rubanyi, 1993). Consequently, there is an

increase in the expression of endothelial cell leukocyte and platelet adhesion molecules, endothelial permeability (Ross, 1999), endothelial cell death and detachment (Woywodt *et al.*, 2002) and reduction in repair (Vasa *et al.*, 2001; Hill *et al.*, 2003). This disruption in vascular homeostasis is the first process in atherosclerotic disease (Ross, 1999) with the severity of the disorder predictive of sustaining a future cardiac event (Halcox *et al.*, 2002). The mechanisms of endothelial dysfunction are complex and summarised below (Figure 2.3).

A reduction in NO bioavailability is the most prominent characteristic of endothelial dysfunction. Indeed, lower levels of eNOS expression and NO synthesis have been demonstrated in atherosclerotic lesions from human carotid arteries (Oemar *et al.*, 1998). The loss of NO leaves the endothelium more vulnerable to the progression of atherosclerosis due to its atheroprotective properties (Figure 2.1). Evidence also suggests that an increase in reactive oxygen species (ROS) production is key in the pathophysiology of endothelial dysfunction. Greater concentrations of superoxide anions have been observed in areas of plaque in atherosclerotic coronary arteries (Sorescu *et al.*, 2002) and administration of antioxidants such as vitamin C improve the vasodilatory response to acetylcholine in patients that exhibit endothelial dysfunction (Taddei *et al.*, 1998). A main source of ROS is NADPH oxidase which is activated/upregulated in endothelial cells by oscillatory shear stress, angiotensin II (Ang II) and inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukins (Griendling *et al.*, 2000). ROS reduces NO bioavailability by directly interacting with NO to form peroxynitrite (Beckman *et al.*, 1990) and through oxidation of BH_4 causing eNOS “uncoupling”, where electrons derived from NADPH are added to oxygen rather than L-arginine, resulting in superoxide production instead of NO (Landmesser *et al.*, 2003).

This creates a vicious circle of greater generation of peroxynitrite. Peroxynitrite also causes eNOS uncoupling through oxidation of BH_4 (Milstien & Katusic, 1999) and can oxidise low-density lipoproteins (LDL) in the subendothelium (Darley-Usmar *et al.*, 1992). Oxidised LDL can be ingested by macrophages to form foam cells thus contributing to plaque development (Ross, 1999) and are taken up by endothelial cells causing cell dysfunction (Li *et al.*, 2000). An increase in circulating endothelial cells and microparticles have been observed in patients with atherosclerosis and endothelial dysfunction (Makin *et al.*, 2004; Esposito *et al.*, 2006) and are an indication of cell damage and senescence. ROS can cause endothelial cell senescence by damaging telomeres and impairing telomerase activity [reviewed by Erusalimsky, (2009)]. Finally, ROS increases the potent vasoconstrictor endothelin-1 (ET-1) and the expression of endothelial cell leukocyte and platelet adhesion molecules (Lund, 2010).

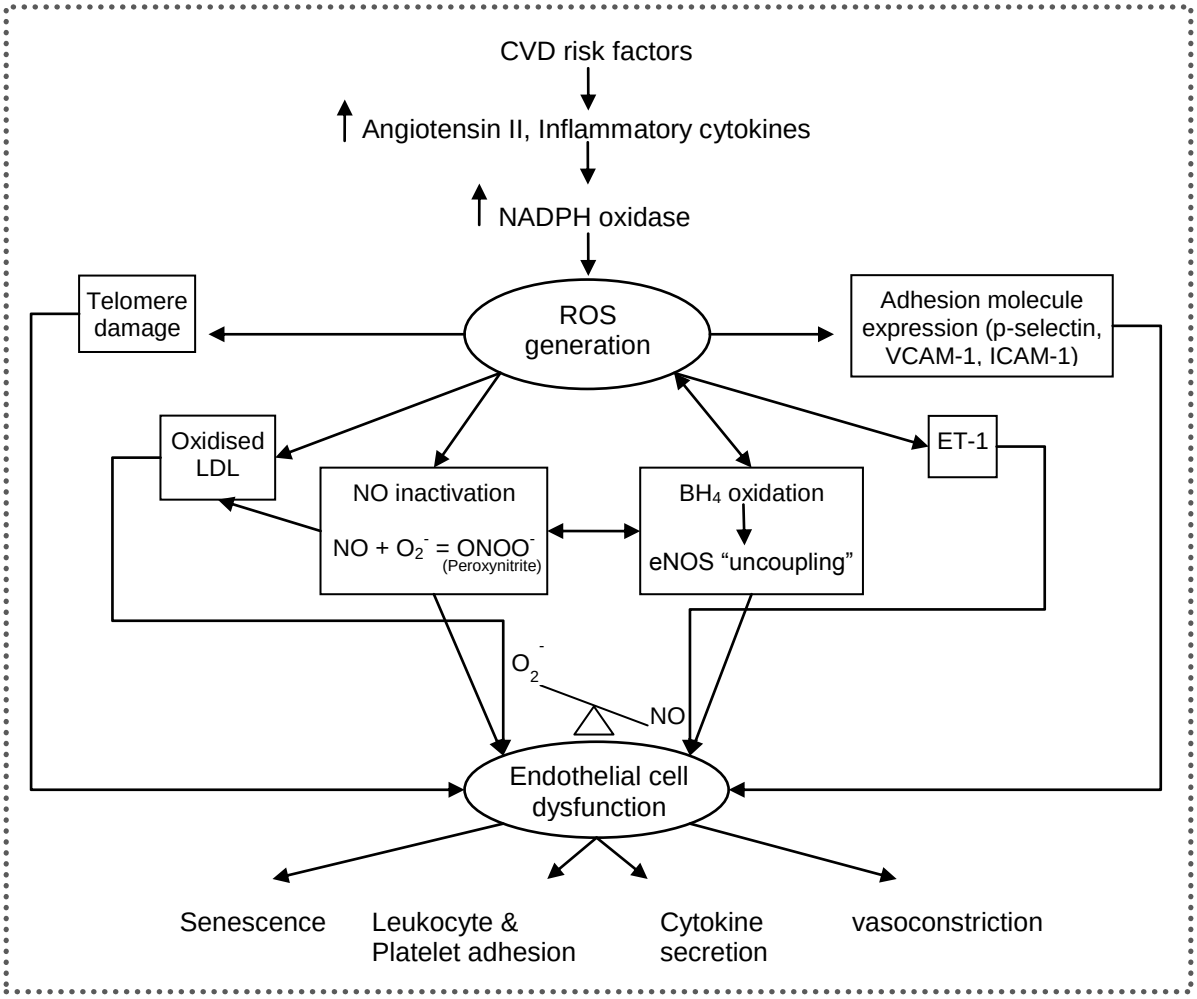

Figure 2.3. The pathophysiology of endothelial dysfunction. An imbalance between reactive oxygen species (ROS) and nitric oxide (NO) is characteristic of endothelial dysfunction. See text for detail. Modified and extensively reviewed by (Cai & Harrison, 2000; Griendling *et al.*, 2000; Landmesser *et al.*, 2004; Lund, 2010). O_2^- = superoxide anion, ET-1 = endothelin-1, BH_4 = tetrahydrobiopterin, LDL = low-density lipoprotein.

2.1.6 Endothelial dysfunction in women across the lifespan

Ageing is a main risk factor for CVD, due in part to the dysfunction of the endothelium. From the age of 40 yrs, brachial artery FMD begins to decline in healthy individuals and at 65 yrs most individuals exhibit an impaired endothelial-dependent vasodilatory response without changes to endothelial-independent dilation (Celermajer *et al.*, 1994). Similarly, endothelial-dependent vasodilation in response to acetylcholine was seen to be significantly reduced with advancing age in both normotensive and hypertensive participants in the

forearm (Taddei *et al.*, 1995) and in the coronary arteries (Egashira *et al.*, 1993). Collectively these findings suggest that ageing is a key mediator of endothelial dysfunction in central and peripheral vessels and can occur in healthy or “at risk” populations. The underlying mechanisms that contribute to the ageing effect on the endothelium include a reduced NO bioavailability (Taddei *et al.*, 2001), endothelial cell telomere length (Aviv *et al.*, 2001), reparative ability of progenitor cells (Heiss *et al.*, 2005) and an increase in vasoconstrictor tone through secretion of ET-1 (Donato *et al.*, 2009), arterial stiffening (refer to section 2.2 for details), oxidative stress (Hamilton *et al.*, 2001), endothelial apoptosis and inflammation (Csiszar *et al.*, 2004).

Throughout the lifespan endothelial function is preserved for longer in females than men. Endothelial-dependent dilation begins to decline at ~41 yrs of age in men but in women this reduction does not occur until ~ 53 yrs. However, the rate of decline is greater in females (0.49 %/year) than men (0.21 %/year), so that the disparity between gender is eliminated by 65 yrs (Celermajer *et al.*, 1994). This pattern of endothelial dysfunction also occurs in response to acetylcholine with a steady decline in the maximal vasodilatory response in men with advancing age, whereas in women the decline accelerates after the menopausal transition [~46-49 yrs, Figure 2.4; Taddei et al., (1996)].

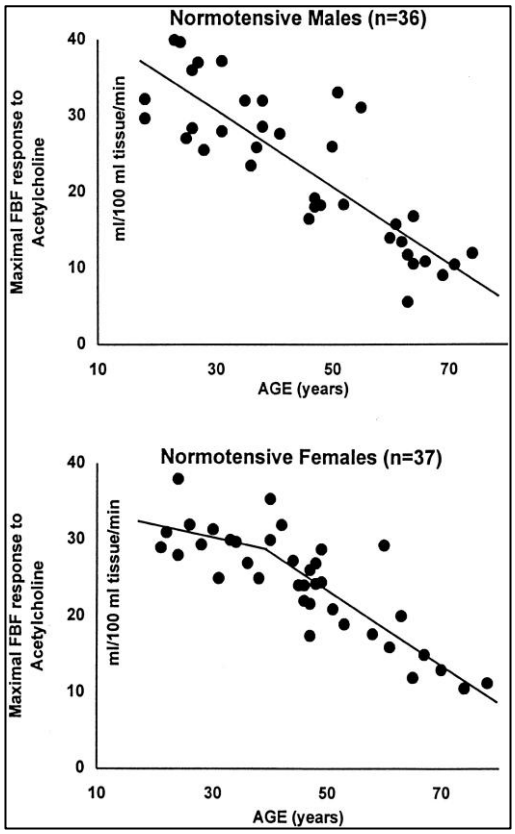

Figure 2.4. The decline in endothelial function with advancing age in healthy males and females. Forearm blood flow (FBF) in response to a maximal dose of intra-brachial acetylcholine infusion (15 µg/100ml forearm tissue/ min) declined with age at a steady rate in men (1.8 %/year). In premenopausal women this decline was less than men (0.5 %/year) but following the menopause at ~49 yrs this decline accelerated at a greater rate (2.2 %/year). Reproduced from Taddei et al., (1996).

The menopause is characterised by a change in hormonal status driven by the loss of oestrogen. Deprivation of oestrogen in women following an ovariectomy significantly impaired endothelial-dependent vasodilation but returned to pre-ovariectomy levels following 3 months of oestrogen replacement therapy (Virdis *et al.*, 2000). Brachial artery FMD declines at each stage of the menopausal transition, with late perimenopausal and postmenopausal women exhibiting a significantly lower FMD than early perimenopausal and premenopausal women (Moreau *et al.*, 2012). Oestrogen aids in maintaining a healthy endothelial function via several mechanisms. Oestrogen binds to oestrogen receptor α (ORα) on the surface of endothelial cells, stimulating binding of ORα with the

p85α subunit of PI3K (Simoncini *et al.*, 2000). PI3K activation stimulates Akt phosphorylation and subsequently eNOS Ser^{1177} phosphorylation and NO production (Haynes *et al.*, 2000). Thus, oestrogen increases NO bioavailability. Ang II increases NADPH oxidase activation and peroxynitrite formation through the Ang II type one receptor [AT_1R; Gragasin et al., (2003)]. Peroxynitrite reduces NO bioavailability (section 2.1.5) and impairs endothelial cell telomerase activity, which enhances cell death (Imanishi *et al.*, 2010). However, in Ang II stimulated endothelial cells, oestrogen treatment impairs NADPH oxidase activation, peroxynitrite formation, and enhances telomerase activity and vasodilation by inhibiting the expression of AT_1R (Gragasin *et al.*, 2003). Therefore, oestrogen attenuates NO degradation and enhances endothelial cell survival (Figure 2.5).

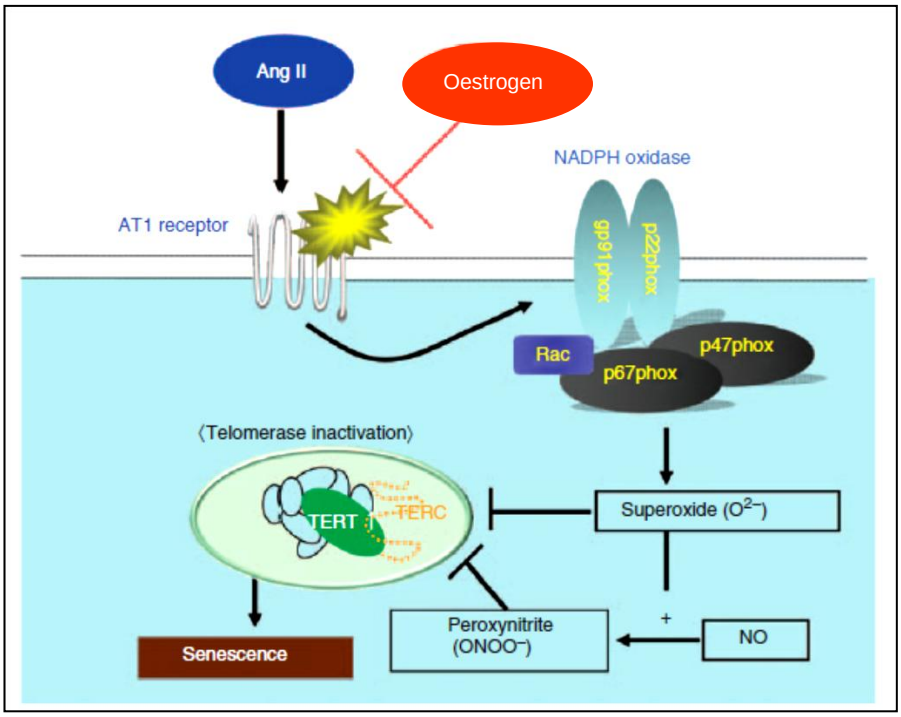

Figure 2.5. The role of oestrogen in reducing endothelial cell senescence. Oestrogen impairs the expression of angiotensin II type one receptor on endothelial cells which in turn reduces the activity of NADPH oxidase and the subsequent formation of peroxynitrite. Therefore, telomerase inactivation is prevented and endothelial cell survival enhanced. Modified from Imanishi et al., (2010).

Finally, oestrogen treatment is associated with lower levels of cholesterol and a higher high-density lipoprotein: low-density lipoprotein (HDL/LDL) ratio due to a greater expression of hepatic LDL receptors which enhances LDL catabolism and reduces the risk of atherosclerosis development (Henriksson *et al.*, 1989; Walsh *et al.*, 1991). The atheroprotective effects of oestrogen and its sudden loss at the menopause may explain the higher vulnerability to fatal cardiac events when compared to men who do not have this protection or accelerated decline in endothelial dysfunction. Indeed a reported ~50% of women who suffered a sudden cardiac death had not previously been diagnosed with coronary heart disease (Bertoia *et al.*, 2012). Furthermore, from ≥40 yrs of age a higher percentage of women than men will die within 1 and 5 years after their first MI or experience a further MI or CV event (Lloyd-Jones *et al.*, 2009).

In premenopausal women, although at a lower risk from CVD than age matched men, endothelial dysfunction is still prevalent due to higher levels of CVD risk factors such as obesity, central obesity and physical inactivity (British Heart Foundation, 2010). Indeed, in premenopausal women with type II diabetes, the protective effect from oestrogen is lost so that the magnitude of endothelial dysfunction is similar to that of age-matched men with the disorder (Steinberg *et al.*, 2000). Obesity and overweight is the 5th cause of global deaths, affecting 35% of women worldwide (World Health Organisation, 2011) and is strongly associated with endothelial dysfunction. An impaired endothelial-dependent vasodilation in women with greater body mass index (BMI) and central fat distribution has been reported (Perticone *et al.*, 2001) but can be significantly increased following weight loss partly due to an increase in NO bioavailability (Pierce *et al.*, 2008). The mechanisms contributing to endothelial dysfunction in obesity involve the secretion of inflammatory cytokines such as TNF-α and

interleukin-6 (IL-6) from adipose tissue, increased adhesion molecule expression on endothelial cells such as p-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), oxidised LDL, oxidative stress and reduced NO bioavailability (Ziccardi *et al.*, 2002; Avogaro & de Kreutzenberg, 2005; Pierce *et al.*, 2008). However, many of these factors along with endothelial dysfunction can be improved or reversed with lifestyle interventions such as exercise.

2.1.7 Exercise training and endothelial function

The effects of exercise on endothelial function has been widely studied in many populations. A literature search on Pubmed with the terms “exercise training” and “endothelial function” produced 227 results. A summary of this search in populations with or at risk of CVD are displayed in Table 2.1. The majority of studies in these populations have shown improved endothelial function post-training, either by increased FMD or blood flow response to intra-arterial infusion with acetylcholine. However, some studies have shown no improvements. Motohiro *et al*., (2005) and Kobayashi *et al*., (2003) observed an increase in lower limb endothelial function following exercise training but not in the arm, indicating the exercise had only a local effect on the working limb vasculature. Other studies have observed differences in the exercise effects upon endothelial function between different exercise modes [Table 2.1; McDermott *et al*., (2009)]. Moreover, contrasting results have been observed in healthy young sedentary populations. Moriguchi *et al*., (2005) observed an increased brachial artery FMD post-training in hypertensive adults but not normotensive. Similarly, popliteal artery FMD was improved post lower limb cycling training in young healthy adults (Rakobowchuk *et al.*, 2008) but not brachial artery FMD (Rakobowchuk *et al.*, 2012). Conversely, increases in

upper limb endothelial function in response to lower limb exercise training have been reported in healthy adults in other studies (Kingwell *et al.*, 1997; DeSouza *et al.*, 2000; Birk *et al.*, 2012). These contrasting results might be explained by the differences in the type, volume and intensity of exercise adopted in the study (Goto *et al.*, 2003), the definition of intensity used, or that initial functional improvements might be negated by arterial structural changes (Tinken *et al.*, 2008; Birk *et al.*, 2012). Higher intensity exercise training programmes may negate improvements (Goto *et al.*, 2003) or indeed impair endothelial function (Bergholm *et al.*, 1999) due to greater production of ROS. However, the intensity of exercise (70-80% maximal oxygen uptake; $\dot{V}O_{2max}$) adopted in these previous studies has been used in other studies which have reported improvements in endothelial function (Motohiro *et al.*, 2005; De Filippis *et al.*, 2006; Tjønna *et al.*, 2008).

Table 2.1. Effects of exercise training on endothelial function in populations with or at risk of cardiovascular disease.

Author	Participants	Exercise training programme	Results
Edwards *et al*., (2004)	CAD patients	- treadmill and cycling - 12 weeks, 3/week -15-20 min ↑ to 40-50 min -40-50% HR_{max} ↑ to 70-85% HR_{max}	↑ Brachial FMD
Luk *et al*., (2012)	CAD patients	- 8 weeks, 3/week, 1 hr sessions - resistance and aerobic (treadmill, cycling, rowing, steps, arm ergometry)	↑ Brachial FMD
Walsh *et al*., (2003)	CAD patients	- 8 weeks, 2 supervised & 1 home/week - cycling and walking at 70-85% HR_{peak} for 45-60 min	↑ Brachial FMD
Motohiro *et al*., (2005)	Post-MI patients	- 3 weeks, 5/week, 1hr sessions - walking & cycling, HR at 70% $\dot{V}O_{2max}$	↑ Calf ↔ brachial reactive hyperaemia
Katz *et al*., (1997)	CHF patients	- 8 weeks, 3/week, 30 min sessions - handgrip contractions at 70% WR_{max}	↑ Peak forearm reactive hyperaemic response to Ach
Kobayashi *et al*., (2003)	CHF patients	- 3 months, 2-3/week, two 15 min/day - cycling at HR at the lactate threshold	↑ Posterior tibial artery FMD ↔ Brachial FMD
Maiorana *et al*., (2000)	CHF patients	- 8 weeks, 3/week, 1 hr sessions - resistance (55-65% WR_{max}) and aerobic (cycling and walking at 70-85% HR_{peak})	↑ Forearm reactive hyperaemic response to Ach
McDermott *et al*., (2009)	PAD patients	- 24 weeks, 3/week, resistance or aerobic - treadmill exercise, 15 min ↑ to 40 min	↑ Brachial FMD in aerobic group only
De Filippis *et al*., (2006)	Overweight non-diabetic & type II diabetic	- 8 weeks cycling + 30 handgrip contractions every 5 min - 60% $\dot{V}O_{2max}$ - 20 min, 3/week ↑ to - 70% $\dot{V}O_{2max}$ - 45 min, 4/week	↑ Forearm reactive hyperaemic response to Ach
Fuchsjäger-Mayrl *et al*., (2002)	Type one diabetic	- 4 months cycling for ~1 hr, 3/week - 40 min at 60-70% HR_{max}	↑ Brachial FMD
Silva *et al*., (2012)	Metabolic syndrome & type II diabetic	- 6 weeks for 50 min, 4/week - walk/run for 40 min at either 50-60% HR_{max} or 75-85% HR_{max}	↑ Brachial FMD-greatest in higher intensity group
Lavrenčič *et al*., (2000)	Metabolic syndrome	- 12 weeks, 3/ week cycling - 20 min warm-up & 30 min at 80% HR_{max}	↑ Brachial FMD
Stensvold *et al*., (2010)	Metabolic syndrome	- 12 weeks, 3/ week, 3 groups - Interval = 4x4 min at 90-95% HR_{max} separated by 3 min at 70% HR_{max} - Strength = 40-80% WR_{max} - Combined interval (2/week) & strength (1/week)	↑ Brachial FMD in all groups
Mestek *et al*., (2010)	Overweight /obese adults	- 3 months, 5-7/ week, 40-50 min/day, 60-75% HR_{max}	↑ Forearm reactive hyperaemic response to Ach
Sprung *et al*., (2013)	Obese women with PCOS	- 16 weeks, 30 min at 30% HRR, 3/week ↑ to 45 min at 60% HRR, 5/week	↑ Brachial FMD
Higashi *et al*., (1999)	Hypertension	- 12 weeks, 5-7/ week, 30 min brisk walking	↑ Forearm reactive hyperaemic response to Ach
Moriguchi *et al*., (2005)	Hypertension	- 12 weeks, 2/ week - cycling at 50% $\dot{V}O_{2max}$ for 60 min	↑ Brachial FMD in hypertensive ↔ in normotensive
Akazawa *et al*., (2012)	Sedentary postmenopausal women	- 8 weeks, 3/ week, cycling & walking - 30 min at 60% HR_{max} ↑ to 40-60 min at 70-75% HR_{max}	↑ Brachial FMD
Swift *et al*., (2012)	Hypertensive postmenopausal women	- 6 months, 3-4/ week - cycling & walking at 50% $\dot{V}O_{2max}$	↑ Brachial FMD

↑ = increasing, ↔ = no change post-training, CAD = coronary artery disease, MI = myocardial infarction, CHF = chronic heart failure, PAD = peripheral artery disease, PCOS = polycystic ovary syndrome, HR_{max} = max heart rate, $\dot{V}O_{2max}$ = maximal oxygen uptake, HRR = heart rate reserve, WR_{max} = work-rate at maximum contraction, Ach = acetylcholine.

In women, exercise has improved endothelial function in those with obesity (Sprung *et al.*, 2013) and following the menopause (Akazawa *et al.*, 2012; Swift *et al.*, 2012). Although few studies specifically focus on female populations, these studies indicated that exercise, by improving endothelial function, may reduce the risk of CVD. However, more studies are required to identify the optimum exercise regime for enhanced endothelial function in women across the lifespan that present risk factors for CVD. Indeed, given the wealth of studies in the area of exercise and endothelial function, there is no consensus on which type and intensity of exercise is best to yield the greatest benefits, in different populations. The Government recommends that adults in the UK should participate in at least 30 min of moderate-intensity exercise on 5 days/week. However, the type of exercise is not specified and many studies have shown improvements in endothelial function following exercise at a lower frequency per week (Table 2.1). Moreover, the number of adults achieving these guidelines is very low, especially in women with only 4% meeting the government targets (British Heart Foundation, 2012). Consequently, in an attempt to increase adherence, researchers have begun investigating how alternative methods of exercise such as interval exercise, affect vascular health. The advantages of interval exercise are that it is more enjoyable and motivating than continuous type exercise (Tjønna *et al.*, 2008), and produces similar or greater improvements in endothelial function (Table 2.2). Furthermore, interval exercise can be less time-consuming, and given that the main barrier to exercise participation is “a lack of time” (British Heart Foundation, 2012), interval exercise might be a more favourable method of exercising. As shown in Table 2.2 interval exercise protocols are varied. The 4x4 method of interval exercise is perhaps the most widely studied and involves four periods of 4 min at ~90% HR_{max}, followed by active recovery periods of 3 min at 70% HR_{max}

(Wisloff *et al.*, 2007; Schjerve *et al.*, 2008; Tjønna *et al.*, 2008). However, although positive results have been reported in these previous studies, this 4x4 model of interval exercise could perhaps be viewed as 4 periods of short continuous exercise. Given that interval exercise is usually defined as short bursts of activity separated by recovery periods, alternative models of interval exercise have been investigated which might be more enjoyable due to the variable work-rates. Sprint interval training involving 30 s periods of maximal exertion followed by 4-5 min recovery periods, improved endothelial function in the lower exercising limb to a similar degree as longer duration continuous exercise training (Rakobowchuk *et al.*, 2008). However, the effects on systemic endothelial function are unknown and the very high intensity nature of the exercise is likely not appropriate for individuals with or at risk of CVD. Alternative interval exercise programmes involving shorter periods at lower work-rates have improved endothelial function in CAD patients [Table 2.2; Currie *et al*., (2013)]. However, future studies are required to adapt this method of interval exercise training for other populations and investigate and compare the effects on endothelial function with different modes of exercise, in an attempt to identify the optimum exercise type. Additionally, the mechanisms by which interval exercise improves endothelial function needs to be identified as they may differ from that of continuous exercise, potentially due to the different haemodynamics involved during the exercise (i.e. oscillations in work-rate during interval exercise).

Table 2.2. Comparisons between interval and continuous exercise training on endothelial function.

Author	Participants	Interval exercise group	Continuous exercise group	Results
Wisloff *et al*., (2007)	CHF patients	-12 weeks, treadmill, 2/week - 4x4 min at 90-95% HR_{max} separated by 3 min at 50-70% HR_{max}	-12 weeks, treadmill, 2/week - 47 min at 70-75% HR_{max}	↑ Brachial FMD in both groups but greater with interval
Schjerve et al., (2008)	Obese adults	-12 weeks, treadmill, 2/week - 4x4 min at 85-95% HR_{max} separated by 3 min at 50-60% HR_{max}	-12 weeks, treadmill, 2/week - 47 min at 60-70% HR_{max}	↑ Brachial FMD in both groups but greater with interval
Tjonna et al., (2008)	Metabolic syndrome	-16 weeks, treadmill, 3/week - 4x4 min at 90% HR_{max} separated by 3 min at 70% HR_{max}	-16 weeks, treadmill, 3/week - 47 min at 70% HR_{max}	↑ Brachial FMD in both groups but greater with interval
Rakobowchuk et al., (2008)	Healthy adults	- 6 weeks, 3/ week cycling - 4-6 30s sprints followed by 4.5 min recovery periods	- 6 weeks, 5/ week cycling - 40-60 min at 65% $\dot{V}O_{2max}$	↑ Popliteal FMD in both groups similarly
Currie *et al*., (2013)	CAD patients	- 12 weeks, 2/ week cycling -10 x 60s at ~90% HR_{max} separated by 60s recovery at 50w	- 12 weeks, 2/ week cycling - 30-50 min at 51-65% peak power output	↑ Brachial FMD in both groups similarly

↑ = increase post-training, CHF = chronic heart failure, CAD = coronary artery disease, HR_{max} = max heart rate, $\dot{V}O_{2max}$ = maximal oxygen uptake.

2.1.7.1 Mechanisms for exercise-mediated increases in endothelial function

Endothelial function is largely regulated by the balance between pro-atherogenic factors such as ROS and inflammation, and atheroprotective factors such as NO (refer to section 2.1.5). Exercise improves endothelial function by rectifying this imbalance, largely by increasing NO bioavailability. Sessa *et al*., (1994) were the first to observe an increase in NO and eNOS gene expression in the coronary arteries of dogs, following 10 consecutive days of exercise training. Likewise, in humans with CAD, 4 weeks of exercise training (10 min rowing and 10 min cycling performed daily) increased acetylcholine-induced vasodilation in the left internal mammary artery (LIMA), concomitant with increased eNOS mRNA, eNOS protein, eNOS-Ser^{1177} phosphorylation and Akt phosphorylation (Hambrecht *et al.*, 2003). Moreover, greater levels of

plasma nitrate/nitrite were noted following exercise training in postmenopausal women (Zaros *et al.*, 2009) and CAD patients (Edwards *et al.*, 2004). It is proposed that exercise augments eNOS/NO through increased shear stress, which is created by greater arterial blood flow during exercise (Taylor *et al.*, 2002).

The importance of shear stress for increasing endothelial function was evidenced in studies which inflated a blood pressure cuff around one arm during exercise, to prevent increases in exercise-induced shear stress. Following 8 weeks of bilateral handgrip exercise (Tinken *et al.*, 2010) and lower limb cycling exercise training (Birk *et al.*, 2012), brachial artery FMD was only increased in the uncuffed arm, whereas no changes were observed in the artery of the cuffed arm. Shear stress increases NO production by activating PI3K, which subsequently activates protein kinases such as protein kinase A (PKA; Boo *et al.*, 2002), Akt (Dimmeler *et al.*, 1999; Hambrecht *et al.*, 2003) and AMPK (Zhang *et al.*, 2006) that phosphorylate eNOS. Augmented eNOS activation via exercise training may also be aided by a reduction in asymmetric dimethylarginine (ADMA; Schlager *et al.*, 2011), which blocks NO synthesis by competing with L-arginine for its binding site on eNOS (Vallance & Leiper, 2004).

Exercise training also improves endothelial function by upregulating anti-oxidant enzymes and reducing oxidative stress and inflammatory factors (Kojda & Hambrecht, 2005). In CAD patients following exercise training (see Table 2.1 for details) plasma levels of superoxide dismutase (SOD) and NO were increased along with reduced 8-isoprostane, a marker of oxidative stress (Edwards *et al.*, 2004). Likewise, increased SOD protein expression was enhanced in aortic endothelial cells in mice following 3 weeks of exercise training, which was not

observed in eNOS$^{-/-}$ (knockout) mice (Fukai *et al.*, 2000). Thus, activation of eNOS appears important for upregulation of anti-oxidants. Reduced oxidative stress following exercise training is driven by a reduction in the activity of the superoxide generating enzyme NADPH oxidase, and a reduction in the expression of its sub-units (Adams *et al.*, 2005). Furthermore, AT_1R expression in the LIMA was reduced in CAD patients following exercise training which resulted in decreased Ang II- mediated vasoconstriction post-training (Adams *et al.*, 2005). Thus, exercise training reduces ROS partly by downregulating AT_1R expression on endothelial cells, resulting in reduced Ang II-mediated activation of NADPH oxidase and the subsequent generation of superoxide anions. Finally, exercise may improve endothelial cell survival and integrity. In mice following 3 weeks of voluntary running, telomerase activity in the endothelial cells in the wall of the aorta was increased (Werner *et al.*, 2009).

As previously discussed, interval type exercise appears superior for improvements in endothelial function. In comparison to continuous exercise training which had no effect, interval exercise training increased plasma nitrite/nitrate, total antioxidant status and reduced plasma oxidised LDL in patients with chronic heart failure (CHF; Wisloff *et al.*, 2007) and the metabolic syndrome (Tjønna *et al.*, 2008). Similar results were reported in rats with the metabolic syndrome (Haram *et al.*, 2008). The mechanisms behind why interval exercise training improved NO bioavailability to a greater extent than continuous exercise training have not been investigated, although it is suggested that the higher intensity of interval exercise and/or the differences in blood flow and shear stress during the exercise, exerts different effects on the endothelium (Haram *et al.*, 2008; Tjønna *et al.*, 2008). To delineate between these two mechanisms, future studies should match interval and continuous exercise

sessions for intensity and subsequently compare their effects on endothelial function.

2.2 Arterial stiffness in health and disease

2.2.1 Implications of arterial stiffness

Arterial stiffening can be defined as an increased resistance to deformation (Glasser *et al.*, 1997) and is reflected by an increased pulse wave velocity (PWV), intima-media thickness (IMT), pulse pressure (PP) and reduced distensibility. It is a process concomitant with ageing which increases the risk of developing CVD. A faster brachial-ankle PWV which reflects central arterial stiffness is associated with an increased Framingham score, a score that predicts the likelihood of developing coronary artery disease in the next 10 years (Yamashina *et al.*, 2003). Moreover, carotid arterial IMT and distensibility is greater with increasing severity of CVD risk (Simons *et al.*, 1999). This increased risk is driven by atherosclerotic development which is potentiated by arterial stiffness. In post-mortem analysis an increased severity of carotid arterial atherosclerotic plaque was associated with a greater carotid artery β-stiffness index (SI) in patients prior to death (Wada *et al.*, 1994). Furthermore, aortic PWV was significantly increased with an increased presence of carotid and aortic plaques in over 3000 patients enrolled on the Rotterdam study (Van Popele *et al.*, 2001). Atherosclerotic development is believed to be accelerated by arterial stiffening through an increase in pulsatile pressure on the arterial walls. In bovine aortic endothelial cells grown in silastic tubes exposed to steady shear, eNOS phosphorylation was increased in compliant tubes whereas the protein was undetectable in stiff tubes (Peng *et al.*, 2003). Similarly, eNOS phosphorylation was reduced and NADPH oxidase expression increased in the segments of pig carotid arteries which had been cuffed for 24 hrs to reduce

arterial stretch, when compared to the uncuffed segments (Thacher *et al.*, 2010). Therefore, an increased arterial stiffness reduces NO synthesis and increases ROS generation which contributes to endothelial dysfunction and subsequent atherosclerosis as described in section 2.1. Indeed, this relationship was observed in ~300 healthy males (18-81 yrs), in which a strong negative correlation was observed between brachial artery FMD and aortic PWV (r = -0.69) and augmentation index (r = -0.59; McEniery *et al.*, 2006).

Arterial stiffness also increases the risk of CVD mortality. Indeed, in end-stage renal disease patients carotid artery elasticity was a significant predictor of cardiovascular and all-cause mortality (Blacher *et al.*, 1998), and in elderly individuals (70-100 yrs) an increased aortic PWV was an independent predictor of cardiovascular death (Meaume *et al.*, 2001). Moreover, a greater arterial stiffness is associated with an increased likelihood to experience a cardiovascular event such as stroke or an MI. In the Rotterdam study which involved analysis from 1373 adults aged ≥55 yrs, the risk of experiencing an MI increased by 43% for every 1 SD increase in carotid artery IMT (Bots *et al.*, 1997). A main cause for this increased risk is a greater PP which reflects a greater systolic blood pressure (SBP) and lower diastolic blood pressure (DBP). Indeed, a higher PP in hypertensive elderly females was associated with a greater incidence of cardiovascular events in the 3 year follow-up period (Scuteri *et al.*, 1995). The mechanism by which arterial stiffness increases PP and the subsequent risk of developing an MI is described in detail by Nichols and O'Rourke (2005). An increase in arterial stiffness generates a greater magnitude and faster pulse pressure wave which returns to the aorta more quickly, increasing systolic blood pressure, which increases myocardial oxygen requirements. Simultaneously due to the early pulse pressure wave reflection,

diastolic blood pressure is reduced and therefore left ventricular coronary perfusion pressure is also reduced. Consequently, the heart is required to work harder to eject blood but with less coronary blood supply, predisposing the myocardial tissue to ischaemia and increasing the risk of a cardiac event (Nichols & O'Rourke, 2005).

2.2.2 Pathophysiology of arterial stiffness

Arterial stiffness can cause endothelial dysfunction and potentiate atherosclerosis, but it can also be initiated as a result of the onset of endothelial dysfunction and atherosclerosis. Atherosclerotic plaques and vascular damage occur before increases in arterial stiffness, as evidenced in monkeys that were fed a high fat diet for 18 months (Farrar *et al.*, 1984). In the first 6-12 months of the diet, the amount of atherosclerotic legions in the aorta rapidly increased and subsequently plateaued, and aortic IMT gradually increased throughout the 18 month period. However, aortic PWV only increased after 18 months even though atherosclerosis was already evident. The two main factors associated with endothelial dysfunction and atherosclerosis are reduced NO bioavailability and increased oxidative stress (section 2.1), which reduce the ability of the vessel to vasodilate, thus increasing vascular resistance. Indeed, infusion with the NO inhibitor L-NMMA increased aortic augmentation index in healthy males (Wilkinson *et al.*, 2002) and increased aortic PWV and carotid β-SI in older adults (Sugawara *et al.*, 2007). A lack of circulating angiogenic cells (CACs) might also contribute to arterial stiffness as observed by a linear positive relationship between the number of CACs and arterial elasticity index (Tao *et al.*, 2006), and a negative correlation between CAC number and brachial to ankle PWV (Yang *et al.*, 2013). However, the cause-effect of this relationship is

unknown. Other main causality factors associated with arterial stiffness are changes to the structural properties of the vessel wall.

Collagen and elastin are the two main proteins which provide structural support and elasticity to the arterial wall. An altered ratio between the two proteins, driven by an increase in collagen molecules and/or reduction in elastin, reduce the elasticity of the vessel wall. An increase in advanced glycation endproducts (AGEs) is a main mechanisms by which the collagen content of the arterial wall increases. AGEs are produced as a result of cross-linking between sugars such as glucose and long-lived proteins such as collagen, which increases the stiffness of the walls (Singh *et al.*, 2001). In hypertensive adults, plasma AGEs were significantly higher when compared to normotensive individuals, and were positively correlated with aortic PWV and PP (McNulty *et al.*, 2007). Moreover, treatment with the drug ALT-711 which breaks down AGE cross-links, decreased PP and aortic PWV and increased arterial compliance in adults ≥50 yrs with arterial stiffening (Kass *et al.*, 2001). AGEs also contribute to arterial stiffening by augmenting endothelial dysfunction and atherosclerosis. The binding of AGEs to its receptors which are present on VSMCs, endothelial cells and macrophages, activates the transcription factor nuclear factor-kappa B (NF-κB) resulting in increased expression of inflammatory cytokines (IL-6, TNF-α), tissue factor, vasoconstrictors (ET-1) and adhesion molecules such as VCAM-1 (Singh *et al.*, 2001). Similarly, in young and older adults, higher serum AGEs are associated with higher levels of oxidative stress which can reduce NO bioavailability and increase endothelial dysfunction (Uribarri *et al.*, 2007). Another mechanism of arterial stiffening is linked to an increase in matrix metalloproteinases (MMPs). In young and older adults with and without hypertension, serum MMP-9 and MMP-2 levels were correlated with aortic and

brachial PWV (Yasmin *et al.*, 2005). MMPs can degrade elastin fibres in the arterial wall, thus contributing to reduced arterial compliance (Jacob, 2003).

2.2.3 Arterial stiffness in women across the lifespan

The ageing process is the predominant mechanism for arterial stiffening in both genders. With increasing age, arterial compliance reduces and augmentation index (indicator of vascular resistance), blood pressure and PWV increase throughout the arterial tree (Kelly *et al.*, 1989a; Benetos *et al.*, 1993; London *et al.*, 1995; McVeigh *et al.*, 1999; Tao *et al.*, 2004). Throughout each decade of life, central artery distensibility reduces which increases PWV [Figure 2.6 (Benetos *et al.*, 1993; van der Heijden-Spek *et al.*, 2000)]. The implications of this decrease on cardiovascular disease and mortality are described in section 2.2.1. Several structural and functional mechanisms as described in section 2.2.2 are responsible for the increased arterial stiffness caused by ageing. An increase in enzymes which degrade elastin fibres such as MMPs and serum elastase have been reported with advancing age (Yasmin *et al.*, 2005). Similarly, AGEs which strengthen collagen fibres and increase inflammation, oxidative stress and endothelial dysfunction have been seen to be higher in older adults (60-80 yrs) when compared to younger adults [18-45 yrs (Uribarri *et al.*, 2007)]. Additionally, elastin fibres split and fray with age due to fatigue from cyclic stretch over the lifespan (O'Rourke, 1990). Endothelial function decreases with age (refer to section 2.1.6 for mechanisms) which reduces vasodilation, thus increasing vascular impedance. Indeed, in $eNOS^{-/-}$ mice where NO and endothelial function are reduced, PWV was greater than that of wildtype mice (Soucy *et al.*, 2006).

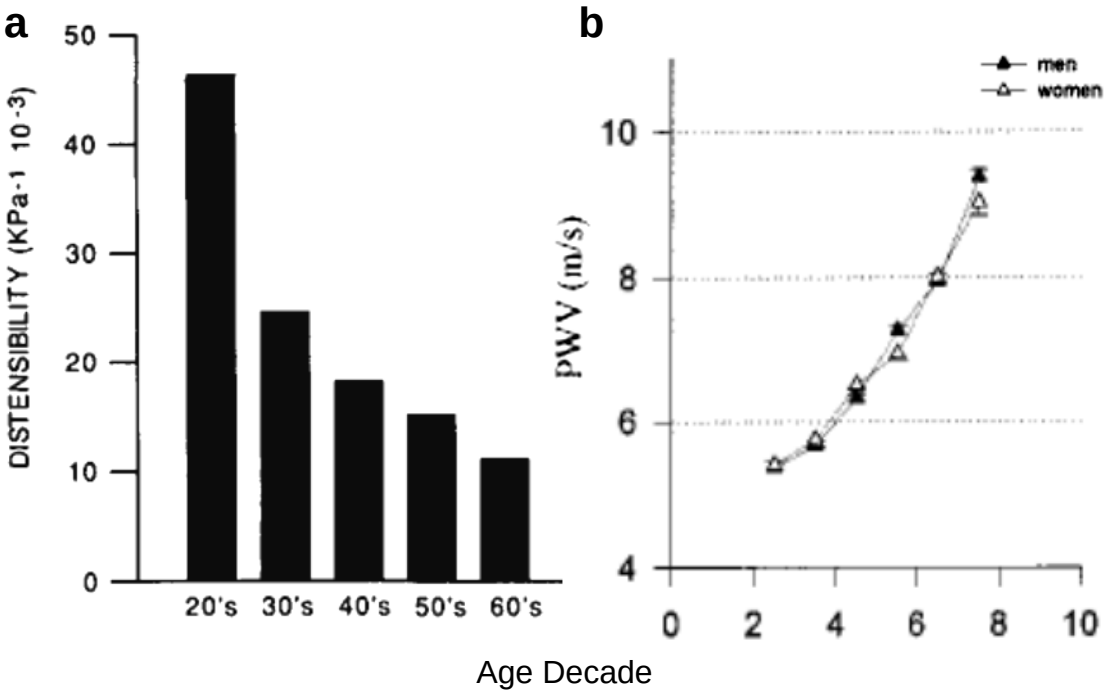

Figure 2.6. The reduction in arterial stiffness across the lifespan. a) Carotid arterial distensibility showed a strong negative correlation with age (r = -0.70) across an age range of 23-73 yrs (Benetos *et al.*, 1993). b) Aortic PWV increases gradually from the second decade of life similarly in men (n = 250) and women [n = 248; van der Heijden-Spek *et al.,* (2000)].

In women, although aortic PWV increases across the lifespan to the same degree as males (van der Heijden-Spek *et al.*, 2000; Smulyan *et al.*, 2001) as shown in Figure 2.6b, carotid artery augmentation index has been reported to be higher in women than males (London *et al.*, 1995; Hayward & Kelly, 1997). The less pronounced effect of oestrogen in the aorta compared to peripheral vessels is likely due to differences in smooth muscle tone and vessel wall structure (van der Heijden-Spek *et al.*, 2000; Zieman *et al.*, 2005). The aorta is less influenced by the vasodilatory effects of oestrogen due to the high elastin fibres and fewer VSMCs in the vessel wall, whereas the muscular peripheral vessels have greater numbers of VSMCs and lower elastin content (Nichols & O'Rourke, 2005). As shown in Figure 2.7a, the gender discrepancy in augmentation index is greater from ≥30 yrs upwards. An increased augmentation index reflects a greater systolic blood pressure and vascular

impedance due to early wave reflection. Given that this has implications for coronary blood supply, it might explain why women have slightly higher prevalence rates for angina pectoris than men (Roger *et al.*, 2011). The reason for a greater vascular resistance in women can be explained in part by body height. Augmentation index is inversely related to body height and given that women are generally shorter than men, an earlier pulse wave will be reflected with a greater magnitude due to a shorter distance to the site of reflection [i.e. arterial bifurcation (London *et al.*, 1995; Hayward & Kelly, 1997)]. Furthermore, an increased arterial stiffness in older women is enhanced due to the loss of oestrogen at the menopausal transition, resulting in an increased PP which is higher than age-matched males (Figure 2.7b), thus augmenting left ventricular load (Smulyan *et al.*, 2001).

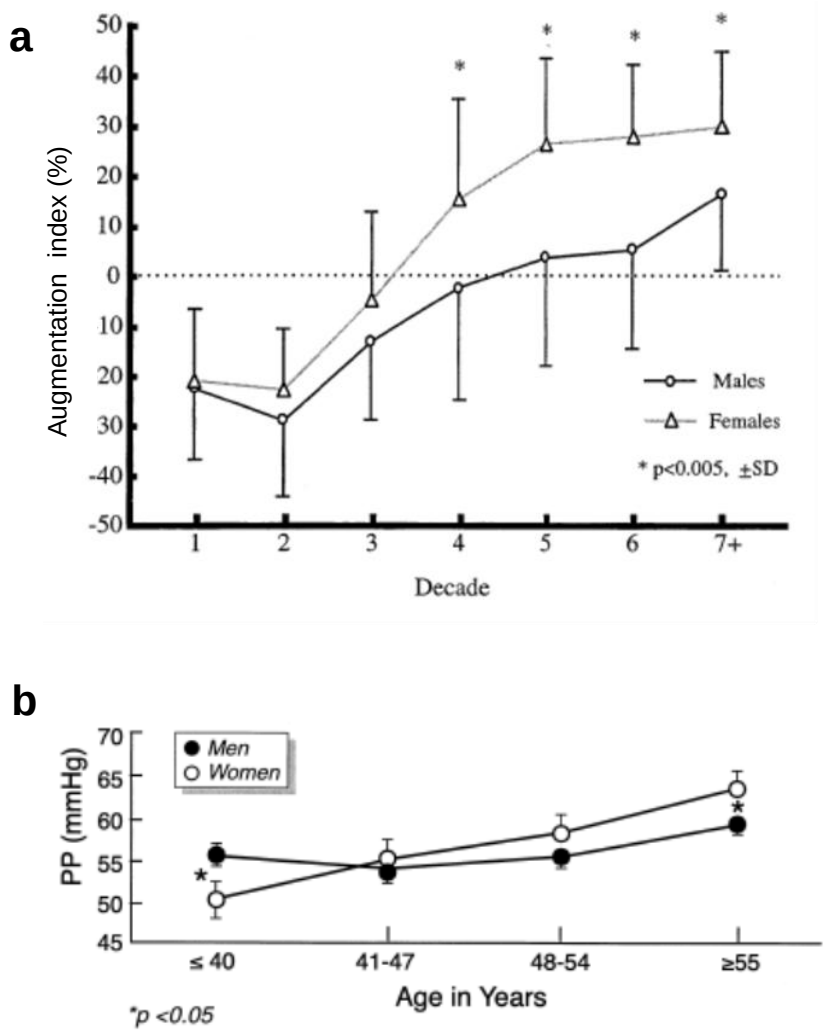

Figure 2.7. Vascular impedance across the lifespan. a) Carotid arterial augmentation index increases across the lifespan but is higher in females than males (Hayward & Kelly, 1997). b) Brachial artery pulse pressure (PP) is greater in men than premenopausal women (≤40 yrs). However, with increasing age, PP is higher in women than men due to a greater number of postmenopausal women within each age group. In the group ≥55 yrs 100% were postmenopausal women (Smulyan *et al.*, 2001).

The loss of oestrogen at the menopause increases endothelial dysfunction which increases CVD risk as explained in section 2.1. The reduction in endothelial function has implications for arterial stiffness (section 2.2.2) which potentiates CVD risk. In age-matched pre and postmenopausal women, carotid arterial distensibility was lower in postmenopausal women, with further reductions with a longer time spent in menopause (Westendorp *et al.*, 1999). Additionally, treatment with hormone replacement therapy in postmenopausal women increased carotid arterial compliance and reduced β-SI (Moreau *et al.*,

2003). These changes in arterial stiffness are related to the physiological actions of oestrogen. Endogenous oestrogen directly affects vasodilation by increasing NO synthesis and enhancing endothelial cell survival due to its effect on telomeres and oxidative stress, through downregulation of AT_1R (section 2.1.6), thus, increasing arterial compliance and reducing vascular resistance. The hormone also has an influence on the structural properties of the arterial wall. Oestrogen receptors have found to be expressed on VSMCs in women (Losordo *et al.*, 1994), and treatment with 17β-estradiol in cultured aortic smooth muscle cells increased elastin and reduced collagen deposition (Natoli *et al.*, 2005). Therefore, the reduction of oestrogen throughout the menopausal transition contributes to arterial stiffening by reducing vascular function and increasing arterial tensile strength.

Factors other than the menopause and age can affect arterial stiffness across a woman's lifespan. Diet and lifestyle behaviour can strongly influence the distensibility of an artery. Obesity is highly prevalent in women and has a negative impact or arterial stiffness. In 660 women aged between 10-86 yrs, aortic PWV was faster with a greater BMI (Zebekakis *et al.*, 2005). However, this relationship was only evident in middle-aged and older women. Similarly, BMI was an independent predictor of aortic PWV only in middle-aged and older women (Wildman *et al.*, 2003). Moreover, obese adults exhibited greater carotid artery IMT and reduced distensibility compared to age-matched non-obese participants (Moore *et al.*, 2013). Furthermore, weight reduction can rectify the increased arterial stiffening as evidenced by a reduction in SBP, DBP, PP, aortic PWV, carotid β-SI and ET-1 following 12 weeks of diet which reduced BMI in adults (Miyaki *et al.*, 2009a). These factors can also be modified through exercise training interventions. The mechanisms by which obesity increases

arterial stiffness are mostly related to an increased endothelial dysfunction associated with obesity as described in section 2.1.6. Figure 2.8 summarises the contributing factors to increased arterial stiffness across the female lifespan.

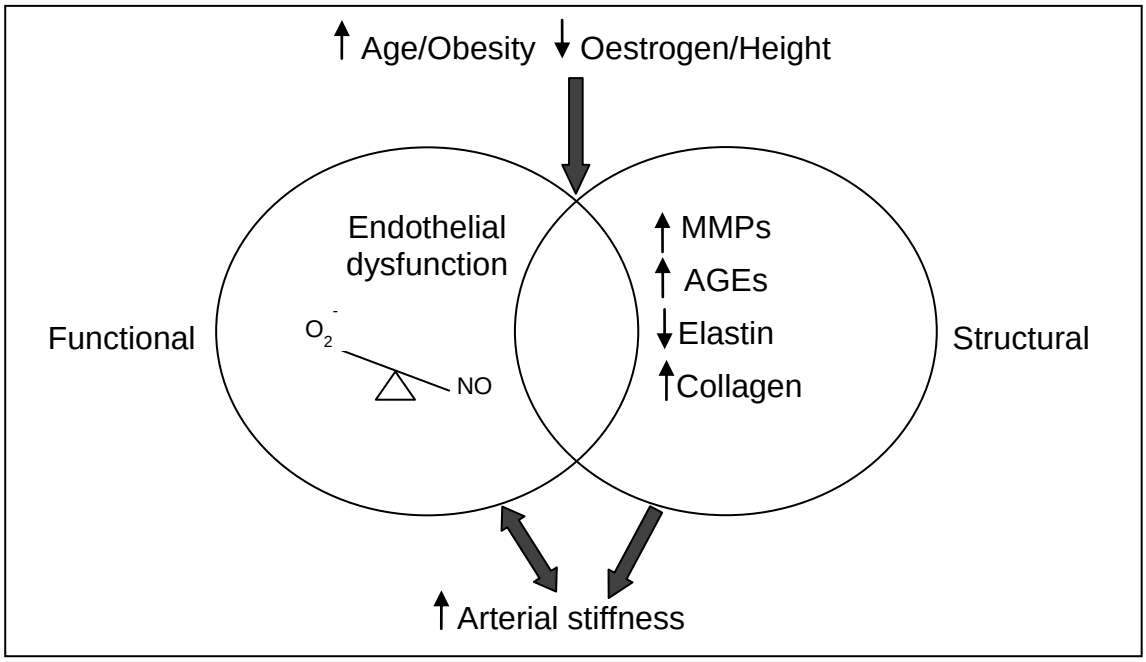

Figure 2.8. Factors and mechanisms contributing to arterial stiffness in women across the lifespan. Age, obesity, oestrogen and height alter the functional and/or structural components affecting arterial stiffness. A change in the structural components can influence the functional component by accelerating endothelial function. A vicious circle is created between endothelial dysfunction and arterial stiffness as arterial stiffness is a cause and consequence of endothelial dysfunction. O_2^- = superoxide anion, NO = nitric oxide, MMPs = matrix metalloproteinases, AGEs = advanced glycation endproducts.

2.2.4 Exercise training and arterial stiffness

Physical activity has favourable effects on arterial stiffness. Significant negative correlations have been observed between $\dot{V}O_{2max}$ and markers of arterial stiffness such as carotid artery augmentation index and aortic PWV (Vaitkevicius *et al.*, 1993). Moreover, an increased carotid artery augmentation index and aortic PWV in postmenopausal women compared to premenopausal women was not observed between physically active pre and postmenopausal women (Tanaka *et al.*, 1998). This indicates that exercising throughout the lifespan may negate the age-related decline in arterial stiffness. Many studies

have examined the effects of exercise training on arterial stiffness in central and peripheral arteries. A literature search on Pubmed with the terms “exercise training” and “arterial stiffness” produced 67 results. Generally, exercise is associated with positive outcomes. Indeed, following continuous aerobic exercise training, carotid arterial compliance increased in postmenopausal women (Moreau *et al.*, 2003) and obese men (Miyaki *et al.*, 2009b); β-SI reduced in postmenopausal women (Moreau *et al.*, 2003; Sugawara *et al.*, 2006) and sedentary adults (Cameron & Dart, 1994), and central PWV decreased in obese women (Yang *et al.*, 2011) and older adults (Vogel *et al.*, 2013). However, other studies have reported no alterations in arterial stiffness. Following 3 months of continuous aerobic exercise training in postmenopausal women (Seals *et al.*, 2001) and sedentary middle-aged and older adults (Tanaka *et al.*, 2002), no changes in aortic or upper limb PWV and carotid artery IMT and augmentation index were evident. Likewise, 8 weeks of cycling exercise training in hypertensive adults had no effect on central or peripheral PWV (Ferrier *et al.*, 2001). These inconsistencies might be related to the exercise type and intensity adopted or the pre-training health of participants, given that individuals with greater arterial stiffness at pre-training experience larger changes at post-training (Rakobowchuk *et al.*, 2013). Additionally, variations in results might be due to a variety of methods that are used to measure arterial stiffness, such as ultrasound imaging and applanation tonometry. Furthermore, there is no consensus on the best type of exercise for improving arterial stiffness. Resistance and strength type exercise may not have as great effect as aerobic type exercise (Bertovic *et al.*, 1999; Miyachi *et al.*, 2004; Rakobowchuk *et al.*, 2005). Moreover, there is growing evidence that interval type exercise might have greater benefits to vascular health than continuous exercise training. As previously reviewed (section 2.1.7) interval

exercise training produced similar or superior effects on endothelial function and appears to be more enjoyable than continuous exercise training (Tjønna *et al.*, 2008). Only a few studies have compared the effects of interval and continuous exercise training on arterial stiffness (Table 2.3). Both sprint interval and continuous exercise training improved arterial distensibility in the lower limb but carotid arterial stiffness measures were unaltered suggesting only local adaptations occurred (Rakobowchuk *et al.*, 2008). Additionally, in hypertensive patients (Guimaraes *et al.*, 2010) and in young women with hypertensive parents (Ciolac *et al.*, 2010), interval exercise training was superior for reducing central PWV. Given the link between greater arterial stiffness and cardiovascular disease (reviewed in previous sections), future studies are required to compare the effects of interval and continuous exercise training on all arterial stiffness markers, in different populations with or at risk of CVD.

Table 2.3. Comparisons between interval and continuous exercise training on arterial stiffness.

Author	Participants	Interval exercise group	Continuous exercise group	Results
Rakobowchuk et al., (2008)	Healthy adults	- 6 weeks, 3/ week cycling - 4-6 30s sprints followed by 4.5 min recovery periods	- 6 weeks, 5/ week cycling - 40-60 min at 65% $\dot{V}O_{2max}$	↑ Popliteal distensibility in both groups ↔ Carotid distensibility or IMT in both groups
Guimaraes *et al*., (2010)	Hypertensive patients	- 16 weeks, 3/ week treadmill - 1 min at 80% HRR and 2 min at 50% HRR for 40 min	- 16 weeks, 3/ week treadmill - 40 min at 60% HRR	↓ Carotid-femoral PWV in interval group only
Ciolac *et al*., (2010)	Young women with hypertensive parents	- 16 weeks, 3/ week treadmill - 1 min at HR at 80-90% $\dot{V}O_{2max}$ and 2 min at HR at LT (50-60% $\dot{V}O_{2max}$) for 40 min	- 16 weeks, 3/ week treadmill - 40 min at HR at 60-70% $\dot{V}O_{2max}$	↓ Carotid-femoral PWV in interval group. Reduced in continuous group but not significantly

↑ = increase post-training, ↔ = no change post-training, HR = heart rate, HRR = heart rate reserve, $\dot{V}O_{2max}$ = maximal oxygen uptake, IMT = intima-media thickness and PWV = pulse wave velocity

2.2.4.1 Mechanisms for exercise-mediated reductions in arterial stiffness

The mechanisms by which aerobic exercise training decreases arterial stiffness relate to both functional and structural changes in the arterial wall. Functional changes relate to increases in endothelial function mediated by augmented NO bioavailability (Joyner, 2000). Greater NO and other vasodilators enable the relaxation of VSMCs, thereby allowing a more distensible vessel. Exercise training increases NO bioavailability and endothelial-dependent dilation by increasing shear stress, which upregulates eNOS and downregulates ROS, as reviewed previously in section 2.1.7.1. Furthermore, NO inhibits VSMC proliferation (Rudic *et al.*, 1998), thus, greater NO bioavailability post-training may reduce arterial stiffness by decreasing VSMC hypertrophy and migration into the sub-endothelium (Joyner, 2000). Increases in NO-mediated endothelial-dependent vasodilation can occur as early as 2 weeks from the start of exercise training (Tinken *et al.*, 2008; Birk *et al.*, 2012). However, structural changes in the vessel wall take longer to occur. It is suggested that the increase in arterial pressure during exercise increases stretching of the vessel wall, which may degrade or reduce cross-linking of collagen and elastin fibres in the arterial wall that contribute to increased stiffness (Joyner, 2000). Indeed, AGEs which are related to protein cross-linking were reduced in plasma following exercise training in obese rats (Boor *et al.*, 2009). Moreover, the content of fibrous proteins in the vessel wall can be altered through exercise training. Following 16 weeks of either running or swimming exercise per day in rats, the elastin content was increased in the aortic vessel wall, along with a reduction in the calcium content in elastin fibres, which was associated with an increased aortic distensibility (Matsuda *et al.*, 1993). The increase in blood flow and pressure experienced in the arterial walls during exercise is the proposed mechanism by which this occurs. Finally, transforming growth factor-β1 (TGF-β1) which

stimulates collagen synthesis is greater in older mice compared with younger mice, but can be reduced following exercise training (10-14 weeks) concomitant with reductions in carotid arterial collagen protein content (Fleenor *et al.*, 2010). Oxidative stress is one regulator of TGF-β1 (Fleenor *et al.*, 2010) thus, reductions in oxidative stress post-exercise training may contribute to greater arterial distensibility by reduced TGF-β1 upregulation and subsequent decreased collagen content.

As reviewed previously, interval exercise training may be superior for decreasing arterial stiffness compared to continuous exercise training (Ciolac *et al.*, 2010; Guimaraes *et al.*, 2010). However, the mechanisms contributing to this observation have not been determined. Ciolac *et al*., (2010) reported a reduction in PWV concomitant with an increase in plasma nitrite/nitrate and reduction in ET-1 in the interval training group only. Therefore, interval exercise training may alter vasomotor tone by tipping the balance between vasomotor factors towards an environment where vasodilators predominate. Potentially, a higher exercise intensity associated with interval exercise produced greater shear stress during exercise, thus, providing a greater stimuli for NO bioavailability (Ciolac *et al.*, 2010; Guimaraes *et al.*, 2010). An alternative hypothesis suggests that the fluctuations in blood flow accompanying interval exercise is a more potent stimuli for functional or structural adaptations, compared to continuous exercise of steady elevated blood flow (Tordi *et al.*, 2010). Thus, future studies are required to match interval and continuous exercise sessions for intensity, to determine whether the exercise intensity or the blood flow profile of the exercise is the most important determinant for arterial stiffness adaptations.

2.3 Circulating angiogenic cells in health and disease

2.3.1 Evidence for the contribution of haematopoietic progenitor cells in vascular repair and angiogenesis

The process of angiogenesis (growth of new blood vessels from existing ones) and the repair of the endothelium from damage/injury, was originally believed to be regulated by the migration and proliferation of existing adjacent endothelial cells in the vessel wall (Clopath *et al.*, 1979). In 1997 this theory was questioned due to the discovery of circulating haematopoietic progenitor cells. Asahara *et al.,* (1997) isolated $CD34^+$ cells (a progenitor cell marker) from human peripheral blood and found that the cells produced NO in response to VEGF and expressed the endothelial cell markers VEGFR-2, CD45 (leukocyte marker) and eNOS. Moreover, these cells formed clusters and tubule structures under culture conditions *in vitro*, and were observed in the capillaries of the ischaemic hind limbs of both mice and rabbits following intravenous infusion. Thus, it was hypothesised that progenitor cells likely mobilised from the bone-marrow, contributed to endothelial repair and angiogenesis. Indeed, subsequent studies identified that the $CD34^+$ cells were of bone-marrow origin using bone-marrow transplantation models. In dogs following bone marrow transplantation, only $CD34^+$ cells with donor DNA had adhered to grafts implanted into the thoracic aorta (Shi *et al.*, 1998). Similarly, in mice following bone-marrow transplant, bone-marrow derived donor cells were localised in the area of ischaemic tissue and formed blood vessels, but were not observed in the non-ischaemic tissues (Tepper *et al.*, 2005). Further evidence for the role of haematopoietic progenitor cells in vascular repair and angiogenesis was provided both *in vitro* and *in vivo*. Progenitor cells cultured from human umbilical cord blood for 7 days were fluorescently labelled and injected into rabbits with a carotid artery injury. After 2 and 4 weeks, fluorescent cells were found on the

neointima of the injured vessel, and the neointima/media was significantly lower than the control group, indicating enhanced endothelial repair by circulating progenitor cells (Hu *et al.*, 2013). In humans, $CD34^+$ cells isolated from umbilical cord blood migrated towards VEGF and stromal-derived factor-1 (SDF-1) and expressed endothelial specific markers E-selectin, VE-Cadherin and von willebrand factor (vWF) when cultured *in vitro* for 2 weeks, suggesting these cells could potentially differentiate into functional endothelial cells (Peichev *et al.*, 2000). Hence, these cells were termed endothelial progenitor cells (EPCs) which sought to maintain vascular homeostasis by repairing endothelial damage.

Since their discovery many studies have attempted unsuccessfully to identify a specific EPC maker. It is likely that these cells represent a heterogeneous population of cells which act collectively to repair vascular damage (Hirschi *et al.*, 2008). Indeed, studies have highlighted the contribution of other types of cells in vascular repair. Mononuclear cells derived from mice spleens expressed $CD34^+$ and VEGFR-2 antigens and formed tubule structures after 14 days of culture in vitro (Werner *et al.*, 2003). Additionally, in patients who had suffered from an acute MI, bone-marrow derived mononuclear cells infused into the ischaemic cardiac tissue, increased stroke volume, myocardial perfusion and left ventricular end-systolic volume to a greater extent than untreated patients (Strauer *et al.*, 2002). The original hypothesis that EPCs contribute to vascular repair by differentiating into mature endothelial cells and incorporating into the endothelial monolayer has been discredited by studies, which have used fluorescent labelling to show that bone-marrow derived cells home to areas of damage but do not integrate into the endothelium. In mice following bone-marrow transplantation of cells expressing green fluorescent protein (GFP^+), the

donor GFP^+ cells accumulated around the ischaemic hind limb but were not incorporated into the vessel (Ziegelhoeffer *et al.*, 2004). Furthermore, in a sophisticated experiment, a segment of carotid artery from wild type mice was denuded and transplanted into the carotid artery of GFP^+ bone-marrow transplanted mice (Hagensen *et al.*, 2012). After 4 weeks, the area was reendothelialised with GFP^+ cells. However, when the injured segment was surrounded by uninjured segments from the wild type mice, the reendothelialised cells were positive for the wild type mice and not the recipient GFP^+ cells. Thus, providing evidence that the endothelial cells adjacent to the injury were responsible for the endothelial recovery. Moreover, *in vitro* culture of mononuclear cells reveal different subpopulations. Mononuclear cells cultured for 4-7 days are commonly referred to as early EPCs but >90% of cells express specific monocyte/macrophage markers (CD144, CD11) and only ~5% express the progenitor cell antigen CD34 (Rehman *et al.*, 2003). These cells secrete growth factors and cytokines and increase perfusion recovery from hind limb ischaemia, but cannot form tubules or incorporate into a HUVEC monolayer *in vitro* (Rehman *et al.*, 2003; Hur *et al.*, 2004). In contrast, late outgrowth EPCs which appear after ~7-21 days of culture display a cobblestone morphology similar to mature endothelial cells, increase perfusion recovery from hind limb ischaemia, and can form tubule structures *in vitro* but do not secrete growth factors or ingest bacteria (Hur *et al.*, 2004). However, it is unknown whether these cells appear *in vivo* or only under specific conditions *in vitro*. Nevertheless, collectively these findings suggest that bone-marrow derived progenitor cells and mononuclear cells contribute to vascular repair and angiogenesis by homing to areas of damage or ischaemia, and act in a paracrine manner by secreting growth factors and cytokines that stimulate the proliferation and migration of residing endothelial cells. Potentially, a small

population of these cells may have the ability to differentiate into mature endothelial cells and become incorporated into the vessel walls. Thus, from herein the cells will be given the universal term of circulating angiogenic cells (CACs) as recommended (Rehman *et al.*, 2003).

2.3.2 Mobilisation, homing and adhesion of circulating angiogenic cells

To enable the repair or growth of blood vessels by CACs, the cells must first be released from the bone marrow into the circulation. Although the mechanisms are not fully understood, it is known that factors such as ischaemia, exercise, oestrogen and cytokines such as VEGF, SDF-1, granulocyte- colony stimulating factor (G-CSF), granulocyte monocyte- colony stimulating factor (GM-CSF), and erythropoietin (EPO), stimulate the mobilisation of CACs into the circulation, where the cells migrate and adhere to sites of vascular injury or stress, and are either incorporated into the vessel wall or secrete growth factors that aid in the proliferation of existing mature endothelial cells [Figure 2.9, Urbich & Dimmeler, (2004)]. In quiescent conditions, the progenitor cells are attached to stromal cells resident in the bone marrow via adhesion molecules such as VCAM-1 (Lapidot & Petit, 2002). The stromal cells influence the microenvironment by releasing SDF-1 so that bone marrow derived CACs reside in the bone marrow via interaction with the SDF-1 receptor CXCR4 which is expressed on CACs. (Lapidot & Petit, 2002). Upon activation by physiological stresses, proteinases such as elastase, cathepsin G and MMPs cleave the adhesive bonds between the stromal cells and CACs, thus releasing the CACs into a permissive niche (Aicher *et al.*, 2005). One of the main pathways is via MMP-9. Through experiments involving MMP-$9^{-/-}$ mice and MMP-9 inhibition, it was revealed that activation of MMP-9 by cytokines cleaved membrane bound kit ligand (mKitL) on stromal cells, allowing release of soluble kit ligand (sKitL) which interacts

with CAC adhesion molecules, thus allowing the release of CACs from the bone marrow (Heissig *et al.*, 2002). The enzyme eNOS also appears to play a pivotal role in CAC mobilisation. In $eNOS^{-/-}$ mice, MMP-9 activity and CAC mobilisation was reduced in response to VEGF, and recovery from hind limb ischaemia was impaired even when $eNOS^{-/-}$ mice underwent bone marrow transplantation using wild type mice cells (Aicher *et al.*, 2003). The release of NO through activation of eNOS in stromal cells in the bone marrow activates MMP-9 and the release of sKitL, which degrades the interaction between SDF-1 and its receptor CXCR4 on CACs, creating a chemotactic gradient that forces CACs to diffuse from the bone marrow into the circulation (Lapidot & Petit, 2002; Aicher *et al.*, 2005).

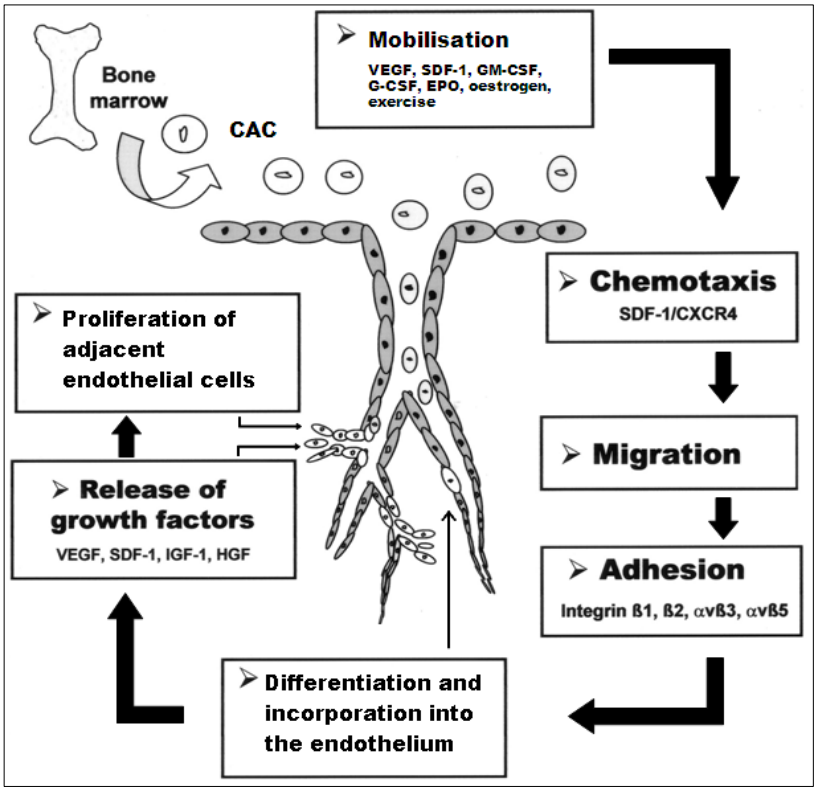

Figure 2.9. The multi-step mechanisms by which circulating angiogenic cells (CAC) aid in vascular repair and angiogenesis. Cytokines and other factors mobilise CACs from the bone marrow niche into the circulation through a chemotactic gradient. The cells migrate and adhere to the activated vasculature where the CACs either differentiate into mature endothelial cells and become incorporated into the endothelium, or the cells secrete growth factors which aid in angiogenesis of vascular repair by the proliferation and migration of existing functional endothelial cells in the vessel wall. Adapted from Urbich & Dimmeler (2004).

Following injury or physiological stress, the endothelium becomes activated causing aggregation of platelets which in combination with activated endothelial cells, secrete VEGF and SDF-1 (Zampetaki *et al.*, 2008). CACs once released from the bone marrow migrate towards this stimulus due to the expression of the VEGF and SDF-1 receptors, VEGFR-2 and CXCR4, respectively. SDF-1 is a strong homing chemokine as evidenced by both *in vivo* and *in vitro* studies. An increase in plasma SDF-1 in mice augmented CAC mobilisation and migration from the bone marrow in a dose dependent manner (Hattori *et al.*, 2001). In healthy humans, cultured CACs migrated towards SDF-1α in a boyden chamber assay *in vitro* in a dose dependent manner, which was

impaired following CXCR4 receptor inhibition (Zheng *et al.*, 2007). The migration of CACs might act partly via a PI3k/Akt/eNOS pathway as inhibition of PI3K and eNOS activation by wortmannin and L-NAME, respectively, inhibited CAC migration towards SDF-1α *in vitro* (Zheng *et al.*, 2007). SDF-1 can also bind to its receptor CXCR7 which is present on CACs and contributes to cell survival, endothelial adhesion and transendothelial migration (Dai *et al.*, 2011). eNOS may also play a role in CACs functional ability to migrate and aid in angiogenesis in the vasculature, as the intravenous infusion of bone marrow derived cells from $eNOS^{-/-}$ mice into both $eNOS^{-/-}$ and wild type mice, impaired perfusion recovery from hind limb ischaemia (Aicher *et al.*, 2003). Furthermore, monocyte chemoattractant protein-3 (MCP-3) which is secreted from macrophages at the sites of vascular damage contributes to CAC homing, as MCP-3 increased CAC migration *in vitro* and stimulated the development of microvessels in a matrigel plug implanted in mice *in vivo* (Bousquenaud *et al.*, 2012). MCPs can also stimulate the adhesion of CACs to the endothelium by activating the β_1-integrin expressed on CACs (Fujiyama *et al.*, 2003). Adherence to the activated endothelium is a requirement for CACs to exert their angiogenic effects which involves the interaction between integrins on CACs and adhesion molecules on the vasculature (Urbich & Dimmeler, 2004). β_2-integrin is expressed on CACs (Chavakis *et al.*, 2005) along with other integrins such as $\alpha_4\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ (Urbich & Dimmeler, 2004). Integrins and ligands such as P-Selectin Glycoprotein Ligand-1 (PSGL-1) on CACs interact with adhesion molecules and ligands such as ICAM-1, E-selectin and P-selectin, which are upregulated on the activated endothelial cells (Zampetaki *et al.*, 2008). Enhanced adhesion of CACs is produced by stimulation of CACs by high mobility group box 1 (HMGB1) which is secreted from necrotic endothelial cells (Zampetaki *et al.*, 2008). Figure 2.10 depicts the process of CAC to an injured

endothelium. Although CACs are mobilised in response to acute physiological stress such as following an MI (Shintani *et al.*, 2001) or after an exercise session (Laufs *et al.*, 2005), CAC mobilisation and function are impaired in individuals who are in a state of chronic stress or inflammation as reviewed in the next section.

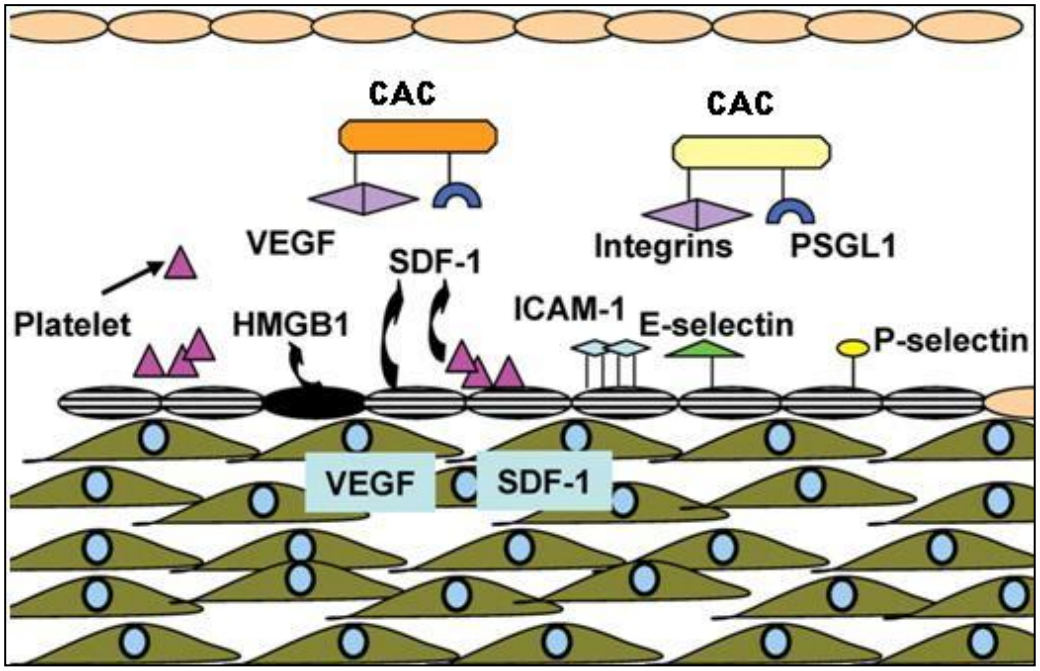

Figure 2.10. Circulating angiogenic cell (CAC) adhesion to an activated endothelium. Aggregated platelets and activated endothelial cells secrete VEGF and SDF-1 which stimulate the migration of CACs to the site of injury. Integrins and PSGL1 on CACs interact with adhesion molecules ICAM-1, E-selectin and P-selectin on the endothelium which is enhanced by release of HMGB1. Adapted from Zampetaki *et al*., (2008).

2.3.3 Circulating angiogenic cells and cardiovascular disease risk

The number of CACs in peripheral blood can be quantified by flow cytometry using antibodies that detect specific markers on CACs, whilst CAC function is assessed using functional migration, adhesion and colony-forming assays *in vitro* (refer to the general methods, chapter 3, section 3.12 for details). The number and function of CACs are impaired in many disease states. In CAD patients, $CD34^{+}KDR^{+}$ CAC number and the number of colony-forming units (CFUs) and migrated CACs to VEGF was significantly reduced when compared

to age-matched healthy controls (Vasa *et al.*, 2001; Werner *et al.*, 2005). Diabetic patients exhibited reduced CAC tubule formation and adhesion to TNF-α activated HUVECs *in vitro* (Tepper *et al.*, 2002). Additionally, $CD34^{+}CD133^{+}KDR^{+}$ CAC number were 53% lower in diabetic patients when compared to control participants (Fadini *et al.*, 2006c). Low CAC numbers and impaired function are also associated with the presence of CVD risk factors. Indeed, CAC number in peripheral blood and migration, adhesion, CFUs and tubule formation *in vitro,* have been reported to significantly correlate with one of more of the following CVD risk factors; smoking, advancing age, hypertension, family history of CAD, obesity, hyperlipidaemia, and higher values of LDL, plasma glucose, HbA1c, C-Reactive protein (CRP), SBP, triglycerides, carotid artery IMT, and waist circumference (Vasa *et al.*, 2001; Tepper *et al.*, 2002; Hill *et al.*, 2003; Schmidt-Lucke *et al.*, 2005; Werner *et al.*, 2005; Fadini *et al.*, 2006a; Fadini *et al.*, 2006b; Jialal *et al.*, 2010a). Furthermore, CAC number and function are independent predictors of morbidity and mortality. In stable and unstable CAD patients, CAC number predicted the severity of atherosclerotic disease progression with patients with the lowest number of CACs more likely to suffer from a cardiovascular event (Schmidt-Lucke *et al.*, 2005). Moreover, low CAC numbers were an independent predictor of death from cardiovascular related causes, and low CFUs increased the risk of experiencing a first major cardiovascular event in CAD patients (Werner *et al.*, 2005).

2.3.3.1 Mechanisms for CAC impairment in CVD

The low numbers of CACs in CVD populations are believed to be due to defective mobilisation from the bone marrow and/or reduced cell survival in the circulation (Figure 2.11). An imbalance in mobilisation factors such as VEGF and G-CSF may contribute to reduced release of CACs from the bone marrow, by reducing the activation of proteinases which cleave the bonds between

CACs and stromal cells (Jialal *et al.*, 2010b). This impairment in the bone marrow niche may be augmented by reduced eNOS activation, which is required for activation of MMP-9 (Aicher *et al.*, 2003). Reduced eNOS expression and phosphorylation and increased uncoupled eNOS is characteristic of many CVD states (Förstermann & Münzel, 2006). Indeed, in cultured CACs from type 2 diabetic patients, ROS production was greater and CFUs and SDF-1α mediated migration was lower compared to non-diabetics (Thum *et al.*, 2007). However, this impairment was reversed with NOS inhibition. Moreover, in rat bone marrow CACs, NOS inhibition blocked superoxide anion production, providing further evidence that uncoupled eNOS and increased oxidative stress impairs CAC mobilisation (Thum *et al.*, 2007). Attenuated CAC migration to SDF-1 observed in diabetic patients (Thum *et al.*, 2007) and CAD patients (Walter *et al.*, 2005) suggests that the chemotactic gradient required for CAC mobilisation is impaired. Potentially, a reduced integrity of the CXCR4 receptor signalling pathway may mediate this observation. Indeed, CXCR4 inhibition blocks SDF-1α mediated migration *in vitro* (Zheng *et al.*, 2007). Furthermore, CXCR4 inhibition reduced Akt and eNOS phosphorylation in SDF-1α treated CACs, suggesting that an impairment between SDF-1/CXCR4 and PI3K/Akt/eNOS pathway contributes to defective mobilisation (Zheng *et al.*, 2007). Reduced expression of CXCR7, a SDF-1 receptor, decreases cell survival, contributing to low CAC number observed in CVD populations (Dai *et al.*, 2011). Indeed in $CXCR7^{-/-}$ mice, 70% died in the first week of birth and exhibited cardiovascular defects such as myocardial degeneration (Gerrits *et al.*, 2008), suggesting that CXCR7 is critical in maintaining cardiovascular health. CAC survival might be influenced by the physiological redox state. Increased oxidative stress and reduced antioxidant activity, which is characteristic of CVD increases CAC senescence (Case *et al.*,

2008). In CACs of hypertensive rats, an increase in ET-1 receptors augmented ET-1 mediated upregulation of NADPH oxidase, which increased oxidative stress (Chen *et al.*, 2012). Similarly, Ang II which is a pro-atherogenic factor (Lusis, 2000), increases CAC senescence by increasing CAC ROS production and activating apoptosis signalling pathways, thereby reducing the capacity for endothelial repair (Imanishi *et al.*, 2005a; Endtmann *et al.*, 2011). Circulating inflammatory factors often exhibited in disease states such as C-reactive protein [CRP; Verma *et al*., (2004)] and TNF-α (Seeger *et al.*, 2005) also induce CAC cell death. Many of these factors act by altering CAC telomere biology. Oxidised LDL (Imanishi *et al.*, 2004b) and angiotensin II (Imanishi *et al.*, 2005a) reduce telomerase activity in CACs, thereby contributing to diminished cell integrity and greater senescence. Moreover, telomerase activity was attenuated in CACs treated with inhibitors of CXCR4 and PI3K in the presence of SDF-1α, suggesting disruption of the SDF-1α mediated PI3K/Akt pathway reduces CAC survival (Zheng *et al.*, 2010). Finally, it has been postulated that exhaustion of the pool of CACs in the bone marrow is partly responsible for low CAC number in CVD, due to the continuous requirement of CACs to repair vascular damage (Dimmeler & Zeiher, 2004).

Reduced CAC function in the presence of CVD risk factors or in patients with CVD, are caused by many of the same mechanisms responsible for reduced CAC number, including increased oxidative stress, decreased receptor and intracellular pathway activity and attenuated telomerase activity (Figure 2.11). Reduced signalling through CXCR4 mediated pathways impaired CAC migration *in vitro* in CAD patients, and reduced the incorporation and recovery of blood flow in the ischaemic hind limb of mice (Walter *et al.*, 2005). The authors observed that CXCR4 mediated Janus Kinase-2 (JAK-2)

phosphorylation in CACs was reduced in CAD patients, but not CXCR4 expression, indicating that reduced CAC function is mediated by dysfunctional intracellular pathways (Walter *et al.*, 2005). Greater levels of oxidised LDL contribute to atherosclerosis and CVD onset (Lusis, 2000) and influences CAC function. Incubation of oxidised LDL with CACs reduced *in vitro* tubule formation, and downregulated the expression of E-selectin and $\alpha_V\beta_5$-integrin (Di Santo *et al.*, 2008). Moreover, reduced expression or integrity of β_2-integrin reduces transendothelial migration and recovery in mice ischaemic hind limbs (Chavakis *et al.*, 2005). Thus, in chronic inflammation, CAC adhesion, migration and reparative ability might be impaired by circulating factors interacting with adhesion molecules. Finally, reduced telomerase activity not only increases CAC senescence but contributes to CAC dysfunction, evidenced by reduced tubule formation *in vitro* (Imanishi *et al.*, 2004b).

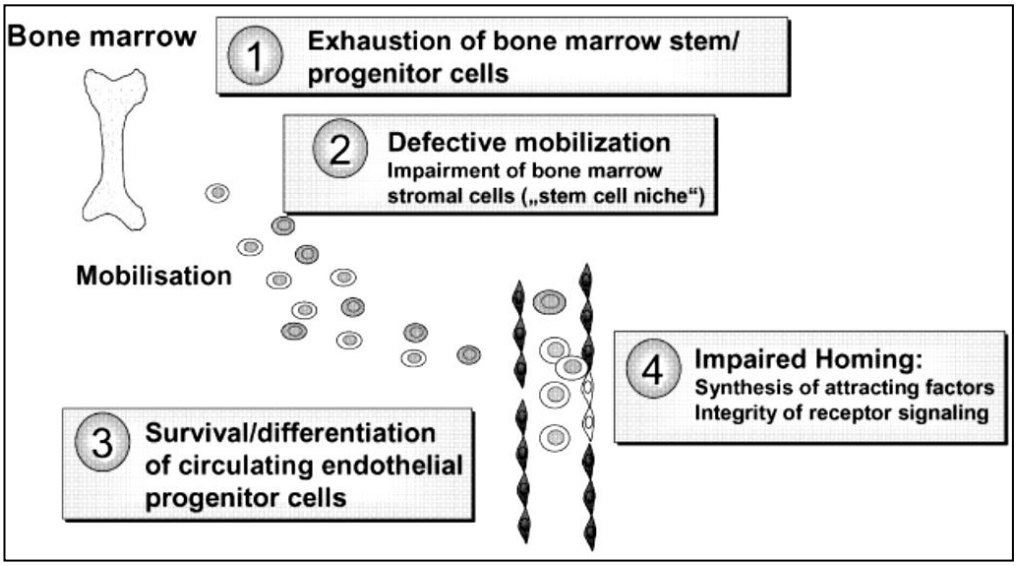

Figure 2.11. Mechanisms contributing to reduced CAC number and function. Increased oxidative stress, angiotensin II, reduced eNOS activity, increased eNOS coupling, decreased mobilisation of cytokines, increased inflammatory factors, reduced telomerase activity and dysfunctional receptor intracellular pathways reduce CAC mobilisation, survival and homing. Reproduced from Dimmeler & Zeiher (2004).

2.3.4 Circulating angiogenic cells in women across the lifespan

Ageing is associated with reduced angiogenic ability as evidenced by attenuated hind limb blood flow perfusion and auto-amputation (i.e. detachment of the limb due to blood vessel damage) following ischaemia in old mice compared to young mice (Rivard *et al.*, 1999). This process may be caused or augmented by the addition of reduced CAC number and function with advancing age. Age is an independent predictor of $CD34^{+}KDR^{+}$ cell number (Vasa *et al.*, 2001) and negatively correlates with CAC number [Figure 2.12; Vasa *et al*., (2001), Schmidt-Lucke *et al*., (2005), Fadini *et al*., (2006b)]. In addition to impaired mobilisation, age also impairs CAC function, including reduced CFUs and *in vitro* VEGF-mediated migration [Figure 2.12; Vasa *et al*., (2001), Werner *et al*.,(2005)].

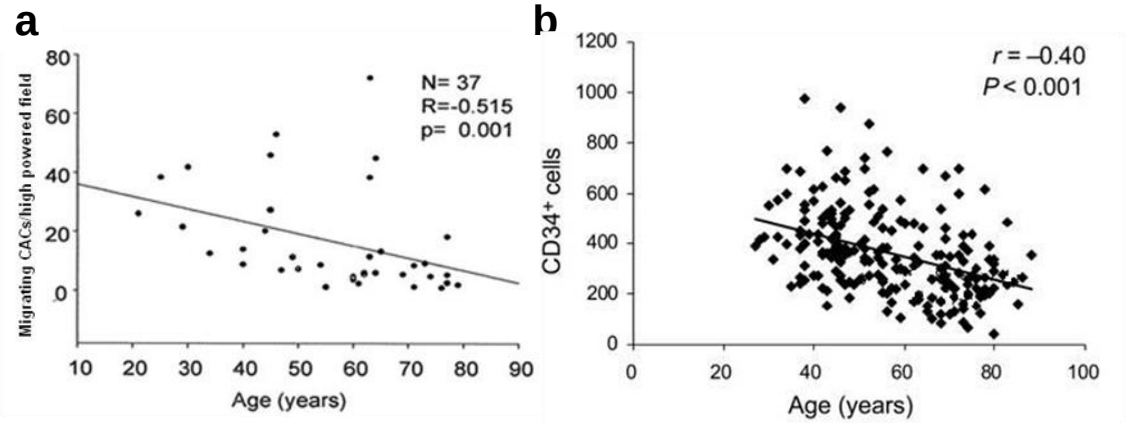

Figure 2.12. Circulating angiogenic cell (CAC) number and function with age. (a) CAC migration to VEGF *in vitro* (Vasa *et al.*, 2001) and (b) the number of circulating $CD34^{+}$ cells (Fadini *et al.*, 2006b) reduce with advancing age.

Many of the mechanisms responsible for impaired CAC mobilisation, survival and function with advancing age are similar to those discussed in section 2.3.3.1 (Figure 2.13) and include exhaustion of the pool of CACs within the bone marrow, increased Ang-II, TNF-α, oxidative stress and reduced pro-angiogenic factors, nitric oxide and eNOS (for review; Williamson *et al.*, 2012).

Additionally, AGEs which accumulate with advancing age (Uribarri *et al.*, 2007) have been shown to reduce *in vitro* $CD34^+$-mediated endothelial sprouting and increase $CD34^+$ cell apoptosis (Scheubel *et al.*, 2006), thus, contributing to reduced CAC survival and function. The expression of Glutathione Peroxidase-1 (GPX1), an anti-oxidant enzyme, in cultured CACs was significantly lower in older adults (65-83 yrs) when compared to younger adults (18-19 yrs), indicating that CACs lose their anti-oxidant defence with advancing age, contributing to greater cell death (He *et al.*, 2009). Moreover, CXCR4 expression on CACs reduce with age and the structure of heparin sulphate on the surface of CACs alters with age (Williamson *et al.*, 2013). These changes correlated with the reduction in migration and homing observed in older adults. Finally, CACs from older adults exhibit reduced telomere length (Kushner *et al.*, 2009) and telomerase activity (Kushner *et al.*, 2011). The abrogation of telomere length appeared from the age of 55 years onwards. These studies were conducted in adult males only, therefore, the time course of the effect of age on CAC telomere biology in women is unknown. However, it is known that oestrogen has an anti-apoptotic and pro-angiogenic effect on CACs, which is lost after the menopausal transition (Imanishi *et al.*, 2010). Therefore, postmenopausal women may experience a loss in CAC number, survival and function that may augment CVD risk, by reducing the capacity for endothelial repair (Figure 2.13). Indeed, $CD34^+KDR^+$ and $CD34^+CD133^+KDR^+$ CACs are higher in pre-menopausal women compared to postmenopausal women (Bulut *et al.*, 2007), where CAC number is similar to that of age-matched older men (Fadini *et al.*, 2008; Rousseau *et al.*, 2010). Although CAC function has not been compared specifically between pre and postmenopausal women, many studies have examined the effects of oestrogen removal and treatment on CAC function *in vitro*. In pre-menopausal eumenorrheic women, the number of

colonies formed from late outgrowth CACs in culture and the number of adhered CACs to a monolayer of HUVECs, was greater compared to age-matched men (Fadini *et al.*, 2008). Moreover, when CACs were incubated with an oestrogen receptor inhibitor, the number of colonies and adherent CACs were significantly attenuated in women only, whereas incubation with oestrogen increased CAC function in men only. Similarly, in rats after ischaemia-reperfusion injury, the percentage increase in capillary density and CACs incorporated or adhered to vessels was greater following infusion of female cultured CACs compared to male CACs (Fadini *et al.*, 2008). Furthermore, *in vitro* CAC apoptosis induced by TNF-α was reduced with simultaneous incubation with oestrogen (Strehlow *et al.*, 2003). The impact of reduced CAC number and function induced by oestrogen loss on vascular repair *in vivo* has been demonstrated in rodent models. In mice following ovariectomy, neointima area and media thickness was greater after carotid arterial injury compared to controls; an effect that was not observed in mice receiving oestrogen treatment (Strehlow *et al.*, 2003). Moreover, following carotid arterial injury, mice which received oestradiol pellets showed larger and faster reendothelialisation than mice receiving placebo pellets (Iwakura *et al.*, 2003).

Oestrogen exerts its effects by activating eNOS predominantly through oestrogen receptor-α and less so through the β-receptor, which is evidenced by impaired CAC function and reendothelialisation in $eNOS^{-/-}$, oestrogen receptor-$\alpha^{-/-}$ and oestrogen receptor-$\beta^{-/-}$ mice following oestrogen treatment (Iwakura *et al.*, 2003; Hamada *et al.*, 2006). Finally, CAC senescence is greater in postmenopausal women due to the effect of and the loss of oestrogen on telomere biology. Oestrogen increases telomerase activity by activating the PI3K/Akt pathway in CACs, triggering upregulation of telomerase reverse

transcriptase, a subunit of telomerase (Imanishi *et al.*, 2005c). Additionally, oestrogen acts by impairing the atherogenic effects of Ang II by downregulating AT_1R expression (Imanishi *et al.*, 2005b) which has previously been described in endothelial cells (section 2.1.6, Figure 2.5). Thus, in postmenopausal women, without the protective effect of oestrogen, CAC number and function are impaired partly by reduced telomerase activity due to reduced PI3K/Akt signalling and increased expression of AT_1R (Imanishi *et al.*, 2010).

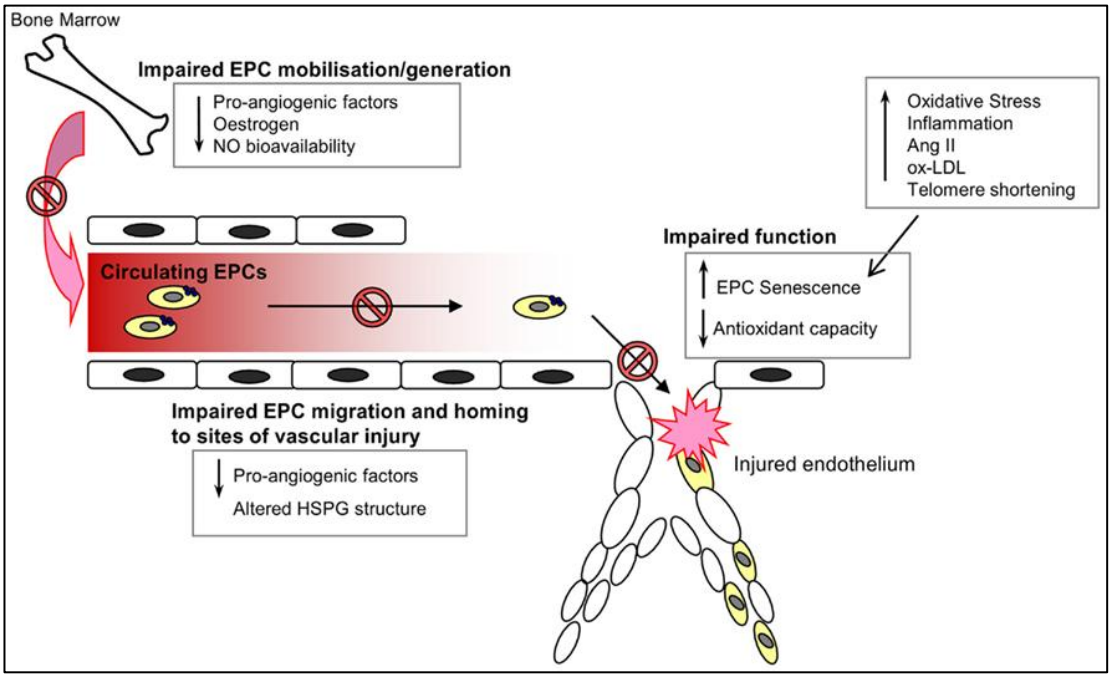

Figure 2.13. Mechanisms contributing to reduced circulating angiogenic cell number and function in women across the lifespan. Reproduced from Williamson *et al*., (2012). EPC = endothelial progenitor cell, NO = nitric oxide, HSPG = heparin sulphate proteoglycans, Ang II = angiotensin II and ox-LDL = oxidised low-density lipoprotein.

As discussed in the previous sections (sections 2.1.6 and 2.2.3), young pre-menopausal women can suffer from reduced vascular repair if other CVD risk factors are present such as obesity. With greater BMI and waist circumference, $CD34^+$, $CD34^+KDR^+$ and $CD34^+CD133^+$ CACs are reduced (Muller-Ehmsen *et al.*, 2008). Moreover, in comparison to age-matched non-obese adults, $CD34^+$ and $CD34^+CD133^+KDR^+$ CACs were significantly reduced in obese adults (>30

kg·m^{-2}) but not in overweight adults [25-30 kg·m^{-2}, MacEneaney *et al*., (2009)]. Similarly, CAC function and angiogenic ability are impaired in obesity. Cultured CACs from obese adults when compared to non-obese, exhibited impaired adhesion to fibronectin, migration, paracrine secretion of IL-8 and MCP-1 and reduced incorporation into tubule structures *in vitro* and into mice vasculature following hind limb ischaemia (Heida *et al.*, 2010). These effects were all reversible following weight reduction (Muller-Ehmsen *et al.*, 2008; Heida *et al.*, 2010). The mechanisms by which obesity reduces CAC number and function relate to a pro-atherogenic milieu created by increased levels of oxidative stress, inflammation (i.e. CRP, TNF-α), reduced anti-oxidant enzyme activity, insulin resistance associated with increased adipose tissue and circulating lipoproteins and triglycerides (MacEneaney *et al.*, 2010; Tobler *et al.*, 2010; Miller-Kasprzak *et al.*, 2011). In combination with reduced CAC number and function, obese women also exhibit greater vascular damage, evidenced by increased levels of circulating endothelial and platelet microparticles (Esposito *et al.*, 2006). This disruption to vascular homeostasis augmented by reduced capacity for vascular repair, contributes to increased CVD risk in obese women. However, vascular homeostasis may be sustained or improved by exercise-mediated increases in vascular repair.

2.3.5 Exercise training and circulating angiogenic cells

Higher levels of physical activity and fitness are associated with better cardiovascular health as discussed previously. A greater capacity to repair vascular damage may contribute to this outcome. Cross-sectional studies have revealed higher numbers of CACs and greater CAC function in physically active adults compared to sedentary controls (Bonsignore *et al.*, 2002). A recent study observed increased $CD34^{+}KDR^{+}$ CACs in endurance trained older men (59-72

yrs) compared to age-matched sedentary men (Yang *et al.*, 2013). However, in young men (21-33 yrs) CAC numbers were the same regardless of fitness level, suggesting that continued exercise throughout the lifespan is necessary to partly negate the age-related decline in CACs and potentially endothelial repair. Nevertheless, CAC migration to VEGF was higher in trained men compared to sedentary counterparts, regardless of age (Yang *et al.*, 2013). Thus, exercise is a readily available method for improving cardiovascular health through increasing the ability for vascular repair. Further evidence is provided by exercise intervention studies which are summarised in Table 2.4.

Table 2.4. Effects of exercise training in humans on circulating angiogenic cell (CAC) number and function in healthy and in diseased populations.

Author	Participants	Exercise training programme	Results	Summary
Laufs *et al*., (2004)	19 CAD patients (70 yrs)	- 4 weeks - Muscle strength training 2-3 days/week - Cycling 3 days/week. 15-20 min at 60-80% $\dot{V}O_{2max}$	- $CD34^+KDR^+$ CACs increased 78 ± 148% - Cultured CAC apoptosis decreased 41 ± 48%	↑ CAC number and *in vitro* survival in CAD patients
Sandri *et al*., (2005)	9 ischaemic PAOD patients (57 ± 8 yrs)	- 4 weeks - Treadmill 6 times daily on 5 days/week - Duration until pain was untolerable	- $CD34^+$ CACs increased 550% - $CD34^+KDR^+$ CACs increased 420% -In vitro integrative capacity increased 160%	↑ CAC number and function in PAOD patients with ischaemia
	9 non-ischaemic PAOD patients (63 ± 7 yrs)	As above -Duration = 75% of maximum walking distance	- $CD34^+$ and $CD34^+KDR^+$ CACs did not change - In vitro integrative capacity increased 205%	↔ CAC number ↑ CAC function in PAOD patients without ischaemia
	15 CAD patients (61 ± 8 yrs)	- 4 weeks - Cycling 6 times daily on 5 days/week - 10 min at 70% HR at $\dot{V}O_{2max}$	- $CD34^+$ and $CD34^+KDR^+$ CACs did not change - In vitro integrative capacity increased 219%	↔ CAC number ↑ CAC function in CAD patients
Steiner et al., (2005)	20 CAD patients (52 ± 10 yrs)	- 12 weeks - Running on 3 days/week for 30-60 min - Plus 2 supervised endurance running sessions/week	- $CD34^+CD133^+KDR^+$ CACs increased 2.9 ± 1.8-fold - No change in $CD34^+$ CACs	↑ and ↔ CAC number depending on definition in CAD patients
Thijssen et al., (2006)	8 older men (67 ± 76 yrs)	- 8 weeks cycling on 3 days/week - 10 min at 65% HRR - 20 min at 65% HRR- increasing by 5% HRR throughout the programme	- $CD34^+CD45^{dim}KDR^+$ CACs reduced by 46% -No change in $CD34^+CD45^{dim}$	↓ and ↔ CAC number depending on definition in older men
Hoetzer *et al*., (2007)	10 middle-aged (36-55 yrs) and older (56-75 yrs) sedentary men	- 3 months walking/jogging - 5-7 days/week for 40-50 min at 60-75% HR_{max}	- VEGF mediated migration increased ~50% - CFUs increased ~120%	↑ CAC function in sedentary males
Sarto *et al*., (2007)	22 CHF patients (61 ± 8 yrs)	- 3 months walking/jogging - 5-7 days/week for 40-50 min at 60-75% HR_{max}	- $CD34^+KDR^+$ CACs increased 151% - CFUs increased 165%	↑ CAC number and function in CHF patients

Erbs *et al*., (2010)	17 CHF patients (61 ± 8 yrs)	-12 weeks cycling - Weeks 1-3 = In hospital 3-6 times daily for 5-20 min at 50% $\dot{V}O_{2max}$ - Weeks 4-12 = home based, 20-30 min at HR at 60% $\dot{V}O_{2max}$, daily - Plus 1 60 min supervised group session	- $CD34^+$ CACs increased 33% - $CD34^+KDR^+$ CACs increased 83% - CAC SDF-1α mediated migration increased 107%	↑ CAC number and function in CHF patients
Sonnenschein *et al*., (2011)	12 metabolic syndrome patients (58 ± 10 yrs)	- 8 weeks cycling 5 days/week for 30 min - Weeks 1-4 = HR at 50% $\dot{V}O_{2max}$ - Weeks 5-8 = HR at 70% $\dot{V}O_{2max}$	- Cultured CACs infused into mice after carotid artery injury increased reendothelialisation in exercise group vs. control - Exercise reduced CAC superoxide and increased nitric oxide production	↑ CAC mediated repair in metabolic syndrome patients
Cesari *et al*., (2012)	47 obese adults (24-69 yrs)	- 3 months walking/jogging - 3 days/week for 45 min at HR at estimated lactate threshold	- $CD34^+$ CACs increased 25% - $CD34^+KDR^+$ CACs increased 50% - $CD133^+KDR^+$ CACs increased 54% - $CD34^+CD133^+KDR^+$ CACs increased 56% -No change in $CD133^+$ and $CD34^+CD133^+$ CACs	↑ and ↔ CAC number depending on definition in obese
Rakobowchuk *et al*., (2012)	20 healthy adults (24 ± 3 yrs)	- 6 weeks interval cycling training (30-40 min) - Moderate intensity group = Repeated 10s at 120% WR_{peak} and 20s recovery - Heavy intensity group = Repeated 30s at 120% WR_{peak} and 60s recovery	- No change in $CD34^+$, $CD34^+KDR^+$ and $CD34^+CD133^+KDR^+$ CACs	↔ CAC number in healthy adults
Nowak *et al*., (2012)	12 intermittent claudication patients (65 ± 9 yrs)	- 12 weeks walking - 3 days/week for 30 min, increasing by 5 min every 2 weeks	- No change in $CD34^+CD45^{dim}CD133^+KDR^+$, $CD45^{dim}CD31^+CD133^+$ and $CD34^+CD45^{dim}CD133^-KDR^+$ CACs	↔ CAC number in intermittent claudication patients
Xia *et al*., (2012)	25 elderly males (68 ± 3 yrs)	- 12 weeks treadmill on - 3 days/week for 30 min	- $CD34^+KDR^+$, $CD133^+KDR^+$ CACs increased - CAC adhesion to TNF-α activated HUVECs increased - Cultured CACs infused into mice after carotid artery injury increased reendothelialisation following exercise - Increased CXCR4 expression, phosphorylated JAK-2 protein and CAC SDF-1α migration	↑ CAC number, function and CAC mediated repair in elderly men
Gatta *et al*., (2012)	14 CHF patients (72 ± 11 yrs)	- 3 weeks. Calisthenics and 30 min cycling twice daily on 6 days/week at 75-85% HR_{max}	- $CD34^+KDR^+$ CACs increased 71% - CFUs increased 51% - Adhesion to TNF-α activated HUVECs increased 50%	↑ CAC number and function in CHF patients

↑ = increase following training, ↓ = decrease following training, ↔ = no change post-training, CAD = coronary artery disease, PAOD = peripheral artery occlusive disease, CHF = chronic heart failure, CFUs= colony-forming units, HR = heart rate, $\dot{V}O_{2max}$ = maximal oxygen uptake, HRR = heart rate reserve, WR = work-rate, VEGF = vascular endothelial growth factor, SDF-1α = stromal-derived factor -1 alpha, HUVECs = human umbilical vein endothelial cells, TNF-α = tumour necrosis factor-alpha and JAK-2 = Janus Kinase-2.

Increases in CAC number and function have been reported in patients with CAD (Laufs *et al.*, 2004; Sandri *et al.*, 2005; Steiner *et al.*, 2005), peripheral artery disease (Sandri *et al.*, 2005), CHF (Sarto *et al.*, 2007; Erbs *et al.*, 2010; Gatta *et al.*, 2012), metabolic syndrome (Sonnenschein *et al.*, 2011) and in obese adults (Cesari *et al.*, 2012) and sedentary middle-aged and older men (Hoetzer *et al.*, 2007; Xia *et al.*, 2012). However, other studies have reported no changes in CAC number (Thijssen *et al.*, 2006; Nowak *et al.*, 2012; Rakobowchuk *et al.*, 2012), which could be due to a different cell type or that a healthy population was studied. Indeed, in healthy adults no changes in CAC number and function have been observed following exercise training (Thijssen *et al.*, 2006; Rakobowchuk *et al.*, 2012). Conversely, increases in CAC number and function in healthy individuals have been reported following acute exercise sessions (Rehman *et al.*, 2004; Laufs *et al.*, 2005; Van Craenenbroeck *et al.*, 2008; Möbius-Winkler *et al.*, 2009; Thorell *et al.*, 2009; Cubbon *et al.*, 2010). Thus, either a greater stimulus is required to evoke long-term changes or that the acute increases in cells are not sustained post-training due to recruitment of cells for endothelial repair, or the cells are degraded as they are not required due to healthy vasculature.

Evidence of the impact of exercise-mediated increases in CAC number and function on endothelial repair and function is provided by studies which have infused human CACs cultured at pre and post-training, into mice following denudation of the carotid artery, and observed faster and greater carotid artery reendothelialisation with infusion of CACs following exercise training (Sonnenschein *et al.*, 2011; Xia *et al.*, 2012). Furthermore, improvements in brachial artery FMD following exercise training have correlated with the exercise-mediated increase in percentage reendothelialisation area in mice (r =

0.61, $p < 0.05$; Xia *et al.*, 2012), and the increase in $CD34^+CD133^+KDR^+$ CAC number ($r = 0.83$, $p < 0.001$; Steiner *et al.*, 2005). This suggests greater CAC number and function contributes to improved endothelial function. Similar findings have been reported in rodents. Compared with sedentary mice, neointima formation was reduced and CAC number and blood vessel growth within a subcutaneously implanted artificial sponge, was greater in mice following 28 days of voluntary running (5100 ± 800 m/24 hrs; Laufs *et al.*, 2004). Additionally, increases in CAC number and CFUs following 10 weeks of swimming (60 min on 5 days/week) in mice correlated with the increase in capillary-to-fibre ratio in the soleus muscle (Fernandes *et al.*, 2012).

2.3.5.1 Mechanisms for exercise-mediated increases in CAC number and function

The mechanisms for exercise-mediated increases in CAC number and function are complex and have not been fully elucidated. eNOS appears important for CAC mobilisation and can be activated during exercise by shear stress and VEGF (Dimmeler *et al.*, 1999; Hambrecht *et al.*, 2003). In $eNOS^{-/-}$ mice and in mice treated with L-NAME, the increase in CACs following 28 days voluntary running exercise was attenuated (Laufs *et al.*, 2004). Similarly, in humans following an acute bout of cycling exercise at 80% of the lactate threshold, the increase in CACs was abolished during infusion with L-NMMA (Cubbon *et al.*, 2010). Moreover, in CAD patients following 12 weeks of running exercise, the increase in CAC number significantly correlated with the increase in plasma nitrate/nitrite levels (Steiner *et al.*, 2005). Activation of eNOS and synthesis of NO in the bone marrow vasculature may release CACs from the stromal cells through the action of MMPs [Figure 2.14; Gielen *et al.*, (2010)]. Indeed, following 3 weeks cycling exercise in CHF patients, tissue inhibitor of metalloproteniases-1 (TIMP-1) was reduced and MMP-2/TIMP-1 and MMP-

9/TIMP-1 ratios were increased (Gatta *et al.*, 2012). Following release from the stromal cells, CACs must exit the bone marrow and home to areas of damage to exert their effects. The major mediator for this process (CXCR4/SDF-1α) is upregulated by exercise. CXCR4 receptor expression on cultured CACs has reportedly increased following treadmill exercise training in PAOD and CAD patients (Sandri *et al.*, 2005) and in sedentary elderly men (Xia *et al.*, 2012). Additionally, SDF-1 levels have also increased following exercise training in CHF patients (Sarto *et al.*, 2007; Erbs *et al.*, 2010). VEGF, which can mobilise CACs from the bone marrow (Asahara *et al.*, 1999) and contribute to CAC homing and migration (Zampetaki *et al.*, 2008) is increased following exercise training (Laufs *et al.*, 2004; Sandri *et al.*, 2005; Sarto *et al.*, 2007; Erbs *et al.*, 2010; Fernandes *et al.*, 2012). VEGF can be activated by hypoxia-inducible factor-1 (Forsythe *et al.*, 1996), which is increased during exercise (Gustafsson *et al.*, 1999). However, other studies have reported no changes in VEGF post-training (Sandri *et al.*, 2005; Steiner *et al.*, 2005). Thus, although VEGF is important, other mediating factors might be required additionally to augment CAC mobilisation and homing.

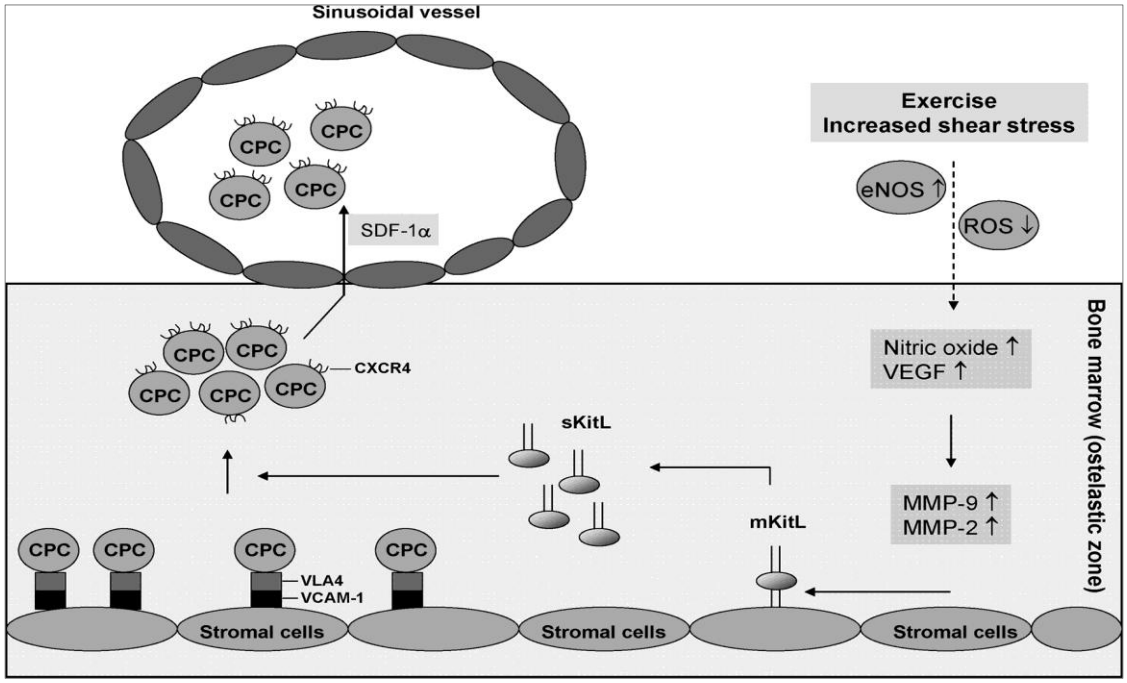

Figure 2.14. Exercise-mediated mobilisation of circulating angiogenic cells. Exercise increases shear stress in the bone marrow vasculature activating matrix metalloproteinases (MMPs) through increased nitric oxide and VEGF. MMPs cleave circulating progenitor cells (CPCs) from the stromal cells through release of soluble kit ligand (sKitL). CPCs exit the bone marrow through a stromal derived factor-1 alpha gradient (SDF-1α) via its receptor CXCR4. mKitL = membrane bound kit-ligand, VLA4 = very late antigen 4, VCAM-1 = vascular cell adhesion molecule-1.

Greater exercise-induced CAC number and function may be mediated by enhanced survival. In humans and mice following exercise training, the rate of apoptosis in cultured CACs was significantly reduced (Laufs *et al.*, 2004), which might be related to telomeres. In mice following 3 weeks of running, telomerase activity in bone marrow derived mononuclear cells was increased (Werner *et al.*, 2009). The authors also observed greater telomerase activity in the bone marrow mononuclear cells of professional runners compared to sedentary individuals. Moreover, enhanced telomerase activity in the endothelial cells of mice aortas post-training was abolished in $eNOS^{-/-}$ mice, suggesting greater NO mediates this effect. SDF-1α activates the PI3K/Akt/eNOS pathway through its receptor CXCR4 on CACs (Zheng *et al.*, 2007) leading to increased telomerase (Zheng *et al.*, 2010). Since, exercise has been shown to increase NO synthesis in CACs (Jenkins *et al.*, 2011; Sonnenschein *et al.*, 2011) and circulating SDF-1

levels (Sarto *et al.*, 2007; Erbs *et al.*, 2010), production of NO via SDF-1 activation of PI3K, may increase CAC survival and function following exercise training. CAC function may also be augmented by reduced oxidative stress. Oxidative stress and inflammation which can impair CAC mobilisation (Thum *et al.*, 2007), activate apoptotic pathways (Imanishi *et al.*, 2005a; Endtmann *et al.*, 2011), inhibit telomerase (Imanishi *et al.*, 2004b; Imanishi *et al.*, 2005a) and interact with CAC integrins and adhesion molecules (Di Santo *et al.*, 2008), is reduced following exercise training. Indeed, reductions in lipid peroxides, ROS production and TNF-α accompanied the increases in CAC number and function following exercise training in CHF patients (Erbs *et al.*, 2010; Gatta *et al.*, 2012) and mice (Fernandes *et al.*, 2012). Similarly, superoxide production from CACs was reduced with exercise training (Sonnenschein *et al.*, 2011).

Further studies are required to understand the molecular mechanisms underpinning the exercise-mediated changes in CAC number and function, which may be influenced by the type, intensity, frequency and duration of exercise. Additionally, these effects may differ according to the population studied. To date, no studies have examined the effects of exercise on CACs specifically in different populations of women (i.e. obese women, postmenopausal women). Moreover, different modalities of exercise such as interval and continuous have not been compared. Only one study has investigated interval exercise training on CAC number (Rakobowchuk *et al.*, 2012) which was in a healthy population and CAC function was not assessed. Furthermore, studies have adopted different methods for defining intensity such as a percentage of HR_{max}, HRR or $\dot{V}O_{2max}$ (Table 2.4). Hence, it is difficult to determine the optimum exercise training programme for improvements in CAC number and function and related functional outcomes for different populations.

Thus, future studies are required to investigate the effects on CACs following specific exercise prescriptions in different populations.

2.4 Summary and thesis aims

In women throughout the lifespan, endothelial function and CAC number and function reduces and arterial stiffness increases due to advancing age, oestrogen loss at the menopause and lifestyle factors that can cause obesity. This imbalance between vascular damage and repair disrupts vascular homeostasis (Figure 2.15), and consequently increases the risk of CVD at older age and in obese women at any age, thereby contributing to the high rates of CVD mortality and morbidity in women.

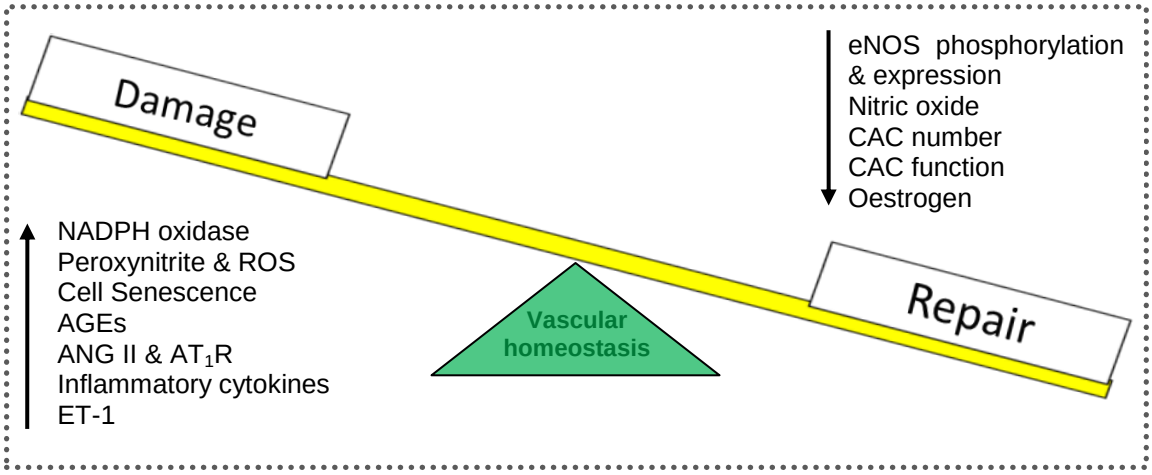

Figure 2.15. The disruption to vascular homeostasis in women across the lifespan. Advancing age, the menopause and obesity increase factors contributing to vascular damage and reduce repair mechanisms and athero-protective factors, collectively increasing the risk of developing CVD. ROS = reactive oxygen species, AGEs = advanced glycation end-products, ANG II = angiotensin two, AT_1R = angiotensin type one receptor, ET-1 = endothelin -1, eNOS = endothelial nitric oxide synthase, CAC = circulating angiogenic cell.

Exercise training interventions can ameliorate these risk factors and rectify the imbalance between vascular damage and repair by increasing eNOS phosphorylation and expression, NO bioavailability, anti-oxidant enzyme activity, telomerase activity, arterial wall elastin content, CAC mobilising factors and reducing AT_1R, oxidative stress and cell senescence. However, no one

study has examined the combined effect of exercise training on endothelial function, arterial stiffness and CAC number and function. Additionally, the optimum exercise type for improving these factors has not yet been determined in women with risk factors for CVD such as observed in obese and postmenopausal women. Interval type exercise which may be more enjoyable and less time consuming, might produce similar or superior effects to continuous exercise in improving endothelial function and decreasing arterial stiffness. However, the effects of interval exercise on these factors has not been compared with continuous exercise in different populations of women. Moreover, this exercise comparison has not been made for CAC number and function per se. Furthermore, in studies in women, interval and continuous exercise sessions have not been matched for exercise intensity, thus, it has not been possible to determine whether the greater physiological stress induced by higher exercise intensity contributed to the beneficial outcomes following interval exercise.

Therefore, the aims of this thesis are as follows:

1. To investigate the effects of exercise on endothelial function, markers of arterial stiffness and CAC number and function collectively, in women across the lifespan (chapters 4, 5, 6);
2. To match interval and continuous exercise for intensity (chapters 4, 5, 6) and work (chapters 4, 6) in order to isolate the work-rate profile during exercise; and
3. To compare the effects of interval and continuous exercise on endothelial function, markers of arterial stiffness and CAC number and function in young, middle-aged obese and postmenopausal women (chapters 4, 5, 6).

Chapter 3 General Methods

3.1 Participant recruitment and screening

Participants were recruited via poster advertisements placed around the local vicinity and email advertisements unless otherwise stated in each chapter. Ethical approval was provided by the University of Leeds Faculty of Biological Sciences Ethics committee which conformed to the Declaration of Helsinki. Inclusion criteria were confirmed at initial contact verbally and/or by email. Exclusion criteria included men, smokers, individuals with known cardiovascular, pulmonary and metabolic disease, musculoskeletal impairments, cancer, contradictions to exercise and if participants had given blood in the previous 3 months or were taking medication. Specific criteria for each population are outlined in the individual chapters. Participants were given at least 24 hours to read through the information sheets and to ask any questions before providing written consent and completing a physical activity readiness questionnaire. This questionnaire was used to further identify any exclusion criteria.

3.2 Experimental procedures

The specific experimental procedures for each study are outlined in the individual chapters. All visits took place on a morning in the temperature controlled exercise physiology laboratories at the University of Leeds. For the vascular assessments and blood collection, participants were instructed not to exercise or consume alcohol in the previous 24 hours and to refrain from consuming food or caffeine in the previous 8 to 12 hours due to their impact on the vasculature (Harris *et al.*, 2010). For the assessment of cardio-respiratory

fitness, participants were instructed to refrain from exercise participation and drinking alcohol and caffeine in the 12 hrs prior to the test.

3.3 Assessment of cardio-respiratory fitness

3.3.1 Ramp incremental step exercise test protocol

Participants performed a seated ramp incremental (RI) and/or a step exercise (SE) test (Figure 3.1) for the assessment of maximal aerobic capacity ($\dot{V}O_{2max}$) and the lactate threshold (LT). These measures enabled exercise-mediated changes in cardiopulmonary fitness to be examined after the exercise interventions and for work-rate during the exercise sessions to be determined. Additionally, the LT was used to delineate between the moderate and heavy-intensity domains (Rossiter, 2011). Participants were seated on an electronically braked cycle ergometer (Excalibur Sport V2.0; Lode BV, Groningen, The Netherlands) and a mouthpiece and nose clip were fitted for breath by breath analyses of pulmonary gas exchange. Prior to commencement of the test, the flow and oxygen and carbon dioxide sensors were calibrated to ensure accurate recording of pulmonary gas exchange (Breeze Suite software V.5.0 and V.7.2, Medgraphics D-series; Medgraphics, Medical Graphics Corporation, St Paul, MN, USA). A rest period (2-4 min) was followed by a period of seated cycling at 20 W (≥ 2 min) before initiation of the RI test, which commenced when gas exchange was at a steady state (i.e. RER = 0.75-0.9) and $\dot{V}O_2$ was stable. The RI rates are specified within each chapter. For the RI test, participants were instructed to maintain a cadence of above 60 rpm until volitional fatigue (determined when the participant could no longer maintain a cycling cadence of at least 50 rpm despite strong verbal encouragement). Heart rate, blood pressure and the Borg's scale of rate of perceived exertion (RPE) were measured every 2 min during the test using a 12-lead ECG,

sphygmomanometer and a visual scale of exertion (6-20) respectively. The work rate at the end of the RI test (WR_{peak}) was calculated as follows:

$$WR_{peak} = RI\ test\ duration\ x\ ramp\ rate + 20$$

Where 20 is the initial 20W of cycling prior to the onset of the ramp.

In chapters 4 and 6, this protocol was modified to include a SE stage. As a plateau in $\dot{V}O_2$ is not usually reached at the end of the RI test, the SE test is used to confirm whether $\dot{V}O_{2max}$ has been achieved (Rossiter *et al.*, 2006). The SE test involved a 5 min period of cycling at 20 W after cessation of the RI test, followed by cycling at 105% of WR_{peak}. During this stage, participants were again encouraged to maintain a high cadence (>80 rpm) until volitional fatigue, determined by a cadence below 50 rpm. This was followed by an active cool-down period (~5 min) of cycling at 20W (Figure 3.1).

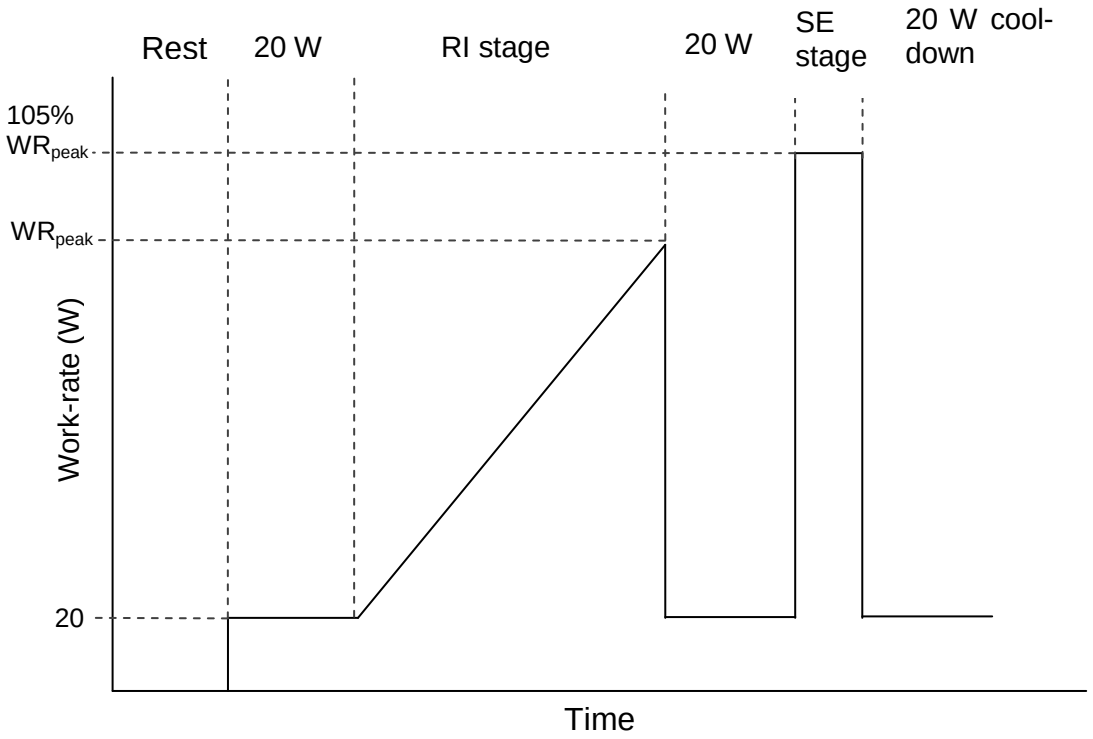

Figure 3.1. Schematic to demonstrate the ramp incremental (RI) step exercise test to determine cardio-respiratory fitness. A rest period was followed by 5 min of cycling at 20 W before the RI stage. This was followed by a 5 min period of cycling at 20 W before the step exercise (SE) stage at 105% of work-rate peak (WR_{peak}). The test ended with a 5 min cool-down at 20 W.

3.3.2 Calculation of $\dot{V}O_{2max}$

Breath by breath data were exported and edited using OriginLab software (OriginPro 8, OriginLab, Northampton, MA, USA). Breaths were eliminated if $\dot{V}O_2$ values fell outside 4 standard deviations around the local mean. As previously described (Bowen *et al.*, 2012) a 12-breath rolling average was calculated and the highest value from both the RI and SE stages of the test defined as the $\dot{V}O_{2peak}$. For chapter 5 only, the $\dot{V}O_{2peak}$ was reported as participants did not complete a SE stage. In chapters 4 and 6 a paired t-test determined that the $\dot{V}O_{2peak}$ values from the RI and SE stages of the test were not significantly different. Therefore, $\dot{V}O_{2max}$ was reported as the average of the RI and SE $\dot{V}O_{2peak}$ values at each time point (i.e. pre, mid and post-training). Relative $\dot{V}O_{2max}$ ($ml \cdot kg \cdot min^{-1}$) was calculated by dividing absolute $\dot{V}O_{2max}$ by body mass (kg).

3.3.3 Estimation of the lactate threshold (LT)

The LT was determined non-invasively using the V-slope method which uses the inflection point of the $\dot{V}O_2$ against $\dot{V}CO_2$ curve as an estimation of the LT (Beaver *et al.*, 1986). This point was further confirmed by a rise in end tidal O_2 and a plateau in end-tidal CO_2 (Whipp *et al.*, 1986). At least two researchers estimated the LT and the average was reported (Figure 3.2).

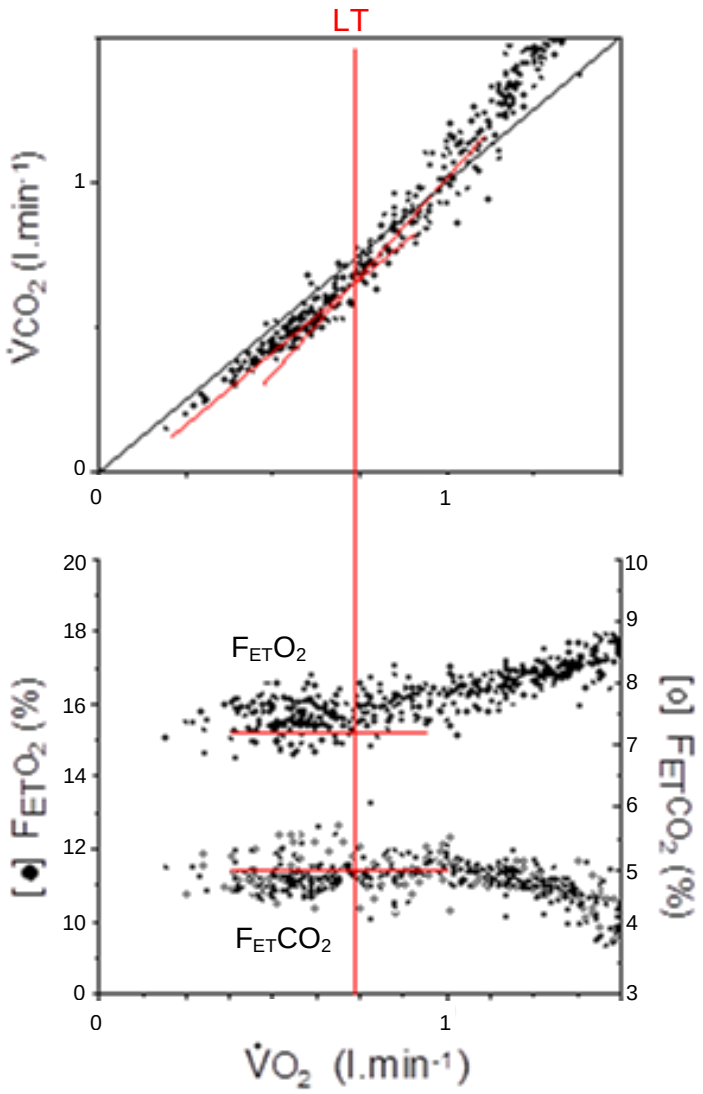

Figure 3.2. Determination of the lactate threshold (LT) using the V-slope method. This point was confirmed by a rise in end-tidal O_2 and a plateau in end-tidal CO_2. $\dot{V}O_2$ = oxygen uptake, $\dot{V}CO_2$ = rate of carbon dioxide elimination, $F_{ET}O_2$ = fraction of oxygen in expired air, $F_{ET}CO_2$ = fraction of carbon dioxide in expired air.

3.4 Assessment of body composition

3.4.1 Body mass index (BMI)

Height was measured to the nearest 0.5 cm using a stadiometer and body mass to the nearest 0.1 kg using manual calibrated scales. BMI was then calculated using the following equation:

$$BMI\ (kg/m^2) = \frac{Body\ mass\ (kg)}{Height^2\ (m)}$$

3.4.2 Waist-hip ratio

Waist and hip circumference were measured to the nearest 0.5 cm using a standard tape measure. Participants were instructed to stand with feet together and arms by the sides of the body while measurements were taken. Waist circumference was measured as the narrowest part beneath the ribs and hip circumference was measured as the widest part of the hip region. Waist to hip ratio (WHR) was calculated using the following equation:

$$WHR = \frac{Waist\ circumference(cm)}{Hip\ circumference(cm)}$$

3.5 Assessment of brachial-artery endothelial function

3.5.1 Principles of flow-mediated dilation (FMD)

An impaired vasodilation of the brachial artery in response to an increase in shear stress is indicative of endothelial dysfunction and can be measured non-invasively by ultrasound (Celermajer *et al.*, 1992). This technique involves inducing reactive hyperaemia by releasing a blood pressure cuff after a 5 min period of forearm occlusion, resulting in an increase in blood flow and shear stress (Corretti *et al.*, 2002). Endothelial cells sense shear stress through various mechanotransducers that lead to eNOS phosphorylation and nitric oxide synthesis, resulting in vasodilation (refer to chapter 2, sections 2.1.3-2.1.4 for further detail). The resultant change in vessel diameter is measured using ultrasound imaging and is thought to be predominantly caused by endothelial cell release of nitric oxide (Doshi *et al.*, 2001); hence brachial artery FMD is used as an indicator of NO bioavailability and indicates a healthier endothelial function.

3.5.2 Ultrasound imaging procedure for collection of brachial artery diameters and blood flow velocity

Assessment of brachial artery endothelial function was assessed according to established guidelines (Corretti *et al.*, 2002; Harris *et al.*, 2010; Thijssen *et al.*, 2011a) after 20min of supine rest. Participants were fitted with an electrocardiograph (ECG) via a three-lead setup which was connected to a SphygmoCor pulse wave velocity (PWV) system (SCOR-Vx, AtCor Medical Pty Ltd, Sydney, Australia; refer to section 3.6 for PWV data acquisition). The ECG trace was outputted to a data acquisition system (Powerlab model ML; ADInstruments, Colorado Springs, Colorado, USA) and recorded using LabChart software (LabChart 7.0, ADInstruments; Figure 3.5). This enabled measurement of resting heart rate calculated as an average over 5 min and for brachial artery images to be recorded at end-diastole (explained below).

The brachial artery of the right arm was imaged longitudinally using a 7 MHz linear array ultrasound probe (Aspen, Acuson; Siemens Medical, Camberley, UK) that was held securely by a clamp which allowed manual micro adjustments to be made. Once an optimal image had been obtained, 20 consecutive images were recorded at end-diastole which was triggered from the peak of the R-wave on the ECG trace using vascular imaging software (Vascular Imager, Medical Imaging Applications, Coralville, Iowa, USA). A 5 min period of forearm ischaemia was created by cuff inflation (> 50 mmHg above systolic blood pressure; SBP) distal to the ultrasound probe at the forearm to occlude arterial flow, and 180 end-diastolic images were recorded consecutively from 30 s before cuff release onwards (~2 min). Placement of the probe from the medial epicondyle was measured using a tape measure to ensure the same portion of the artery was recorded after the intervention. For blood flow velocity and shear rates to be calculated the Doppler audio signals were continuously

recorded with the sample volume gate positioned to include the whole lumen as recommended (Harris *et al.*, 2008). Refer to sections 3.5.4 and 3.5.5 for blood flow velocity and shear rates analysis and calculations. The insonation angle of the ultrasound beam was kept as close to 60° as possible to ensure capture of quality ultrasound images while optimising for Doppler recording. Figure 3.3 displays the setup for this technique.

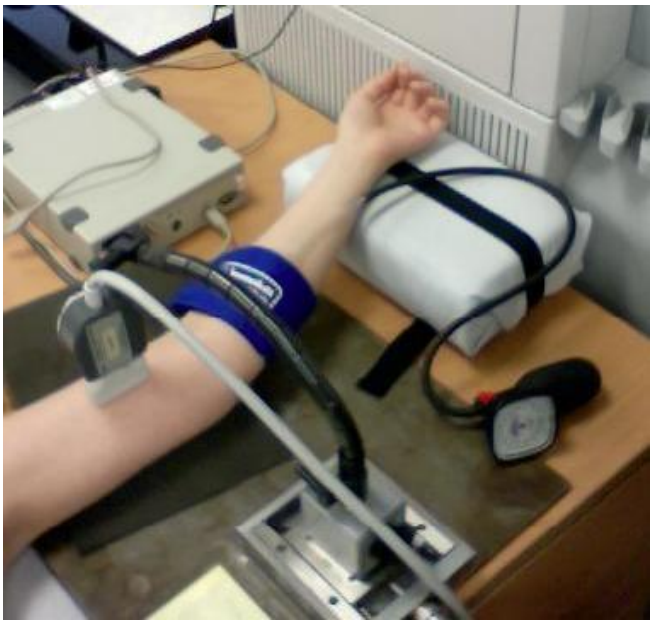

Figure 3.3. Assessment of brachial artery flow-mediated dilation (FMD). Brachial artery images were captured using ultrasound before and after a 5 min period of forearm ischaemia created by inflation of a blood pressure cuff.

3.5.3 Analysis of brachial artery diameter

Image analysis was conducted using edge-detection software (Brachial Tools v.5, Medical Imaging Applications, Coralville, Iowa, USA) to determine brachial artery diameter from far to near-wall tunica media (Figure 3.4). A region of interest was chosen on the part of the artery with the most defined media to media border. Images were manually edited if the diameter confidence interval was less than 70% and rejected if the tunica media wall was not visible.

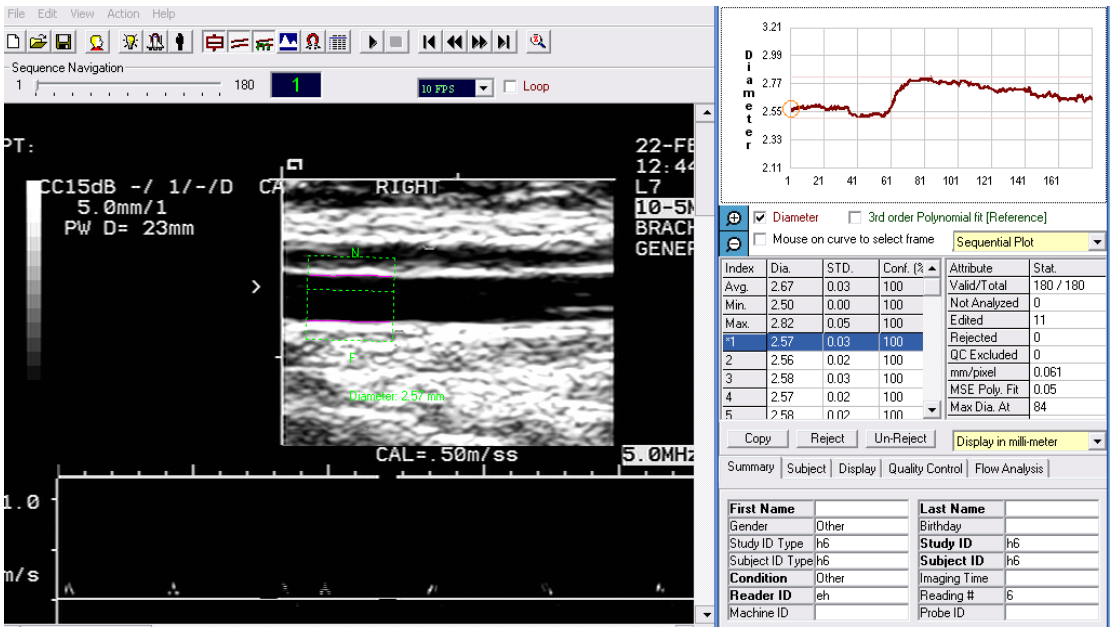

Figure 3.4. Analysis of brachial artery diameter using semi-automated edge-detection software. A region of interest was placed around the part of the artery with the most defined tunica media border. The software measures the distance between the far and near-wall tunica media borders to determine brachial artery diameter.

Resting diameter was calculated from the software as an average of the 20 images recorded prior to cuff inflation. To calculate peak diameter the post cuff release diameters were transferred to Microsoft Excel (Microsoft Office Excel, Redmond, WA, USA) and the highest value from a 3 consecutive cardiac cycle rolling average defined as the peak diameter. Absolute and relative FMD were calculated as follows:

$$\textit{Absolute FMD (mm)} = \textit{Peak diameter} - \textit{Resting diameter}$$

$$\textit{Relative FMD (\%)} = \frac{\textit{Peak diameter} - \textit{Resting diameter}}{\textit{Resting diameter}} \times 100$$

The time from cuff release to peak diameter was calculated using the times recorded on the peak diameter and cuff release ultrasound image frames.

3.5.4 Blood flow velocity analysis

Post cuff deflation blood velocity and shear rates were determined using Fast-Fourier transform to convert the continuous Doppler audio signals to blood

velocity as previously described (Herr *et al.*, 2010). The ultrasound system (Aspen, Acuson; Siemens Medical, Camberley, UK) had two audio outputs with one that encoded blood velocity in the direction of the ultrasound probe and the other for blood velocity travelling away from the probe. These forward and reverse signals were sampled using a data acquisition system (Powerlab model ML; ADInstruments) and recorded on LabChart software (LabChart 7.0, ADInstruments) at a sampling rate of 20 MHz (Figure 3.5).

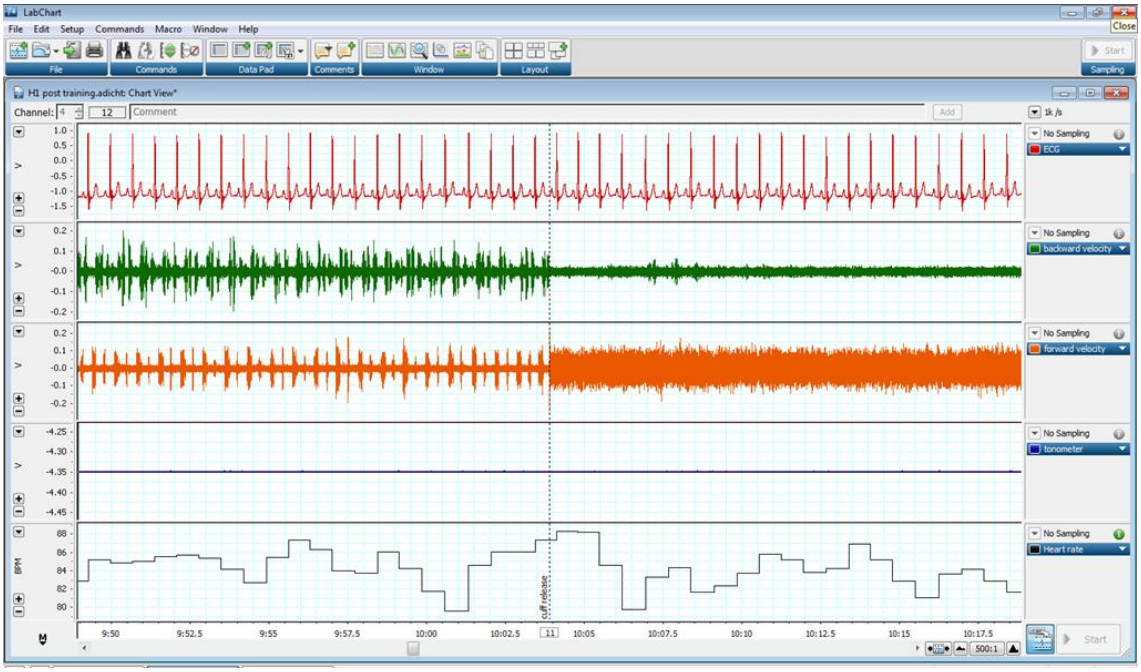

Figure 3.5. LabChart software (LabChart 7.0, ADInstruments) for recording and analysing brachial artery FMD, blood flow velocity and resting heart rate. Signals were recorded at a sampling rate of 20MHz. In order from the top of the screen to the bottom; a continuous ECG was recorded in channel one and backwards and forwards Doppler blood flow from the brachial artery was recorded in channels two and three respectively. The tonometer signals were recorded in channel four (refer to section 3.6) and in channel five heart rate derived from the ECG trace in channel one was determined. The vertical dashed line represents the point at which the blood pressure cuff was deflated during FMD.

Spectral analysis was employed to determine the mean frequency and power (amplitude) of the forward and reverse Doppler signals across 0.1 s intervals from cuff deflation to 2 min post. This data was exported to an Excel

spreadsheet (Microsoft Excel 2007) and the intensity-weighted forward and reverse components of blood velocity were calculated using the Doppler shift equation as follows:

$$\mathrm{u} = (f_d c / 2 f_t \cos\theta)$$

Where u = mean blood flow velocity, f_d = the Doppler shift frequency, c = average velocity of sound in a tissue (1540 m/s), f_t = transmitted frequency, θ = insonation angle. The weighted mean velocity was subsequently calculated using the following equation:

Mean velocity = ((mean forward velocity x forward signal power) - (mean reverse velocity x reverse signal power)) / total power

3.5.5 Calculation of peak blood flow velocity and shear rates

The area under the shear rate curve from cuff deflation to peak dilation (AUC_{peak}) and to 60 s post (AUC_{60}) were calculated from (8 X VTI)/resting brachial artery diameter, where VTI is the velocity time integral for each period as previously described (Rakobowchuk *et al.*, 2012). For chapters 5 and 6, the shear rate AUC and VTI for 90 s from cuff deflation was also calculated, as the time from cuff release to peak diameter increases with age and with the presence of CVD risk factors (Black *et al.*, 2008; Padilla *et al.*, 2009). This equation was used and is recommended as during data acquisition the Doppler sample volume gate width encompassed the whole of the lumen (Harris *et al.*, 2010). The VTI was estimated using the trapezium rule as follows:

$$VTI\ (cm) = \frac{(V_1 + V_2)}{2} \times t$$

Where V_1 and V_2 are consecutive net velocities and t is the time difference between the velocities (0.1 s). Peak reactive hyperaemia (cm/s) was determined as the highest net velocity in the first 10 s post cuff release, and

peak shear rate (s^{-1}) calculated as (8 X peak reactive hyperaemia)/resting brachial artery diameter (Rakobowchuk *et al.*, 2012).

3.5.6 Reliability and validity of the assessment of brachial artery endothelial function

The use of semi-automated edge-detection software is a validated and recommended method for determining brachial artery diameter (Harris *et al.*, 2010). Brachial artery flow-mediated dilation (FMD) exhibits a strong correlation with coronary artery dilation (Takase *et al.*, 1998) and is impaired in participants with risk factors for cardiovascular disease including low oestrogen levels (Lieberman *et al.*, 1994), type 2 diabetes (Henry *et al.*, 2004), smoking (Celermajer *et al.*, 1993) and hypercholesterolaemia (Sorensen *et al.*, 1994). Additionally, FMD has been shown to be predictive of CVD risk with a 13% reduction in the risk of a cardiovascular event occurring for every 1% increase in FMD (Green *et al.*, 2011).

The notion that FMD is largely nitric oxide mediated is evidenced by a study demonstrating attenuated FMD during intra-arterial infusion of the nitric oxide inhibitor N^G-monomethyl-L-arginine (L-NMMA) when cuff placement was distal to the ultrasound probe (Doshi *et al.*, 2001). However, when the cuff was placed on the upper arm proximal to the ultrasound probe, FMD was only partially reduced. The duration of forearm occlusion also impacts on the validity of nitric oxide mediated FMD. A 5 min period of occlusion with L-NMMA infusion abolished FMD where as a 15 min period of occlusion with L-NMMA had no effect on the FMD response, indicating that other factors besides nitric oxide mediated this effect (Mullen *et al.*, 2001). Thus, brachial artery FMD is only indicative of endothelial function and can be used as a prognostic indictor of CVD risk when the cuff is inflated distal to the ultrasound probe for a 5 min

period (Green *et al.*, 2011). These guidelines were adhered to in this thesis in order to maximise validity. The between-day reproducibility from 8 participants assessed on two separate days, four weeks apart (to control for menstrual cycle phase) had a coefficient of variation (standard deviation/average) of 12.4% and an absolute difference of 0.18% for relative FMD and a coefficient of variation of 13.9% and an absolute difference of 0.001 mm for absolute FMD.

Shear stress is a stimulus for endothelial-dependent dilation (section 2.1.3) but shear rate is used as the surrogate measure for the stimulus for brachial artery FMD to eliminate the measurement of blood viscosity (Pyke & Tschakovsky, 2005). However, the portion of the shear rate stimulus post cuff release that mediates the resultant vasodilation is unclear. Shear rate AUC has been shown to have a greater influence on FMD than the peak shear rate (Pyke *et al.*, 2004; Pyke & Tschakovsky, 2007) but the time frame with which shear rate AUC should be calculated remaines undefined. A subsequent study examined the relationship between FMD and the different methods used to quantify shear rate AUC (i.e. peak shear rate AUC, AUC_{peak}, AUC_{30}, AUC_{60}) and reported modest correlations between FMD and the four different shear rate AUC methods in a young healthy population (Thijssen *et al.*, 2009b). However, in children and an older population these correlations did not occur. Therefore, it is recommended that different shear rates are reported but that FMD should only be normalised to shear if specific assumptions are met. These include a significant correlation between FMD and Shear and that the y-intercept of the regression slope between the two variables is zero. Since not all these assumptions were met in this thesis, FMD was not normalised to shear rate AUC. The between-day reproducibility from 5 participants assessed on two separate days, four weeks apart (to control for menstrual cycle phase) are reported in Table 3.1.

Table 3.1. Between-day reproducibility for the different shear rate calculation methods.

	Absolute difference	Coefficient of variation (%)
Peak reactive hyperaemia	3.3 cm/s	12.4
Peak shear rate	29.5 s^{-1}	12.2
Shear rate AUC_{peak}	335 a.u.	18.3
Shear rate AUC_{60}	3203 a.u.	11.5
Shear rate AUC_{90}	2251 a.u.	10.8

3.6 Assessment of pulse wave velocity (PWV)

3.6.1 Recording of pulse pressure waveforms

The velocity of the pulse along an artery is positively correlated with the stiffness of the vessel (Nichols & O'Rourke, 2005) with a faster pulse wave velocity (PWV) associated with greater CVD risk and mortality (Blacher et al., 1999; Yamashina et al., 2003). PWV can be measured non-invasively between two sites in the arterial tree by applanation tonometry (Figure 3.6). This technique involves the flattening (applanation) of the vessel with a pressure sensor probe (tonometer) to eliminate the circumferential wall stresses to allow the intra arterial pressures to be detected (Kelly *et al.*, 1989a; Nichols & O'Rourke, 2005).

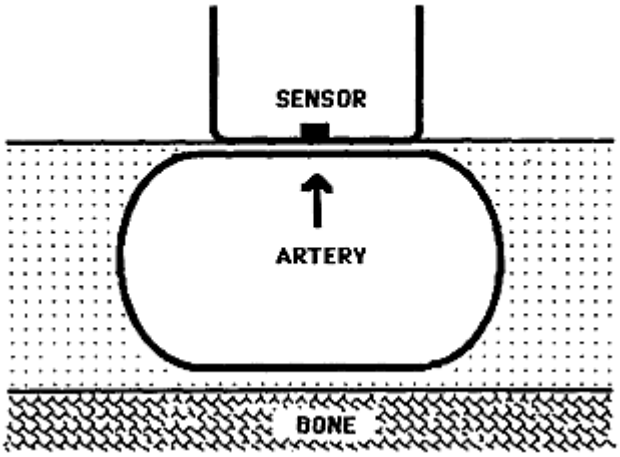

Figure 3.6. Applanation tonometry involves flattening the artery with a pressure sensitive probe to enable intra-arterial pressure to be transmitted. From (Kelly *et al.*, 1989a).

Recordings of arterial pulse pressure waveforms were always acquired after the measurement of FMD therefore, participants were in a relaxed state after at least a 30 min period of lying in a supine position. To determine the carotid to radial PWV (PWV_{cr}) and brachial to foot (PWV_{bf}) at least 30 pulse pressure waveforms were captured at different arterial sites (carotid, radial, brachial and dorsalis pedis arteries), by palpitation of the pulse and placement of the tonometer over the point which displayed the strongest pulse. The distance between the sternal notch to the different arterial sites was measured using a standard tape measure. The distance between the sternal notch and the brachial and radial arteries was measured with the left arm held at a 90° angle to the body. All measurements were acquired whilst the participants were in a supine position with simultaneous ECG recording (refer to section 3.5.2 for ECG setup). Arterial pulse pressure waveforms were recorded using a tonometer (model SPT-301, Millar Instruments Inc., Texas, USA) connected to a SphygmoCor PWV system (SCOR-Vx, AtCor Medical Pty Ltd) which enabled simultaneous ECG and pulse pressure waveform recordings. These signals

were outputted to a data acquisition system (Powerlab model ML, ADInstruments) and recorded using LabChart software (LabChart 7.0, ADInstruments; Figure 3.8).

3.6.2 Analysis of pulse pressure waveforms and calculation of PWV

To determine the most reliable method of calculating PWV_{cr} and PWV_{bf}, three different methods were compared. The first two methods involved the SphygmoCor PWV system and the other method involved analysing tonometer recordings on LabChart. Pulse pressure waveforms and arterial distances were recorded (as above) from 7 participants on two separate days, 4 weeks apart. PWV was calculated as follows:

$$PWV\ (m/s) = \frac{\Delta distance}{\Delta PTT}$$

Where $\Delta distance$ is the difference in distance between the sternum and the two arterial sites and ΔPTT is the difference in pulse transit time between the two arterial sites. The PTT at each arterial site was calculated as the difference in time between the peak of the R-wave on the ECG and the foot of the upstroke on the pulse pressure waveform. Using the SphygmoCor PWV system the PTT was calculated automatically using either the second derivative or the intersecting tangents method (Figure 3.7). For PWV_{cr}, pulse pressure waveforms were recorded for 20 s at the carotid and then the radial artery. The distance between the sternum and these sites were inputted manually and either the second derivative or the intersecting tangent method chosen to calculate the average PTT and the subsequent PWV. This was repeated for PWV_{bf}.

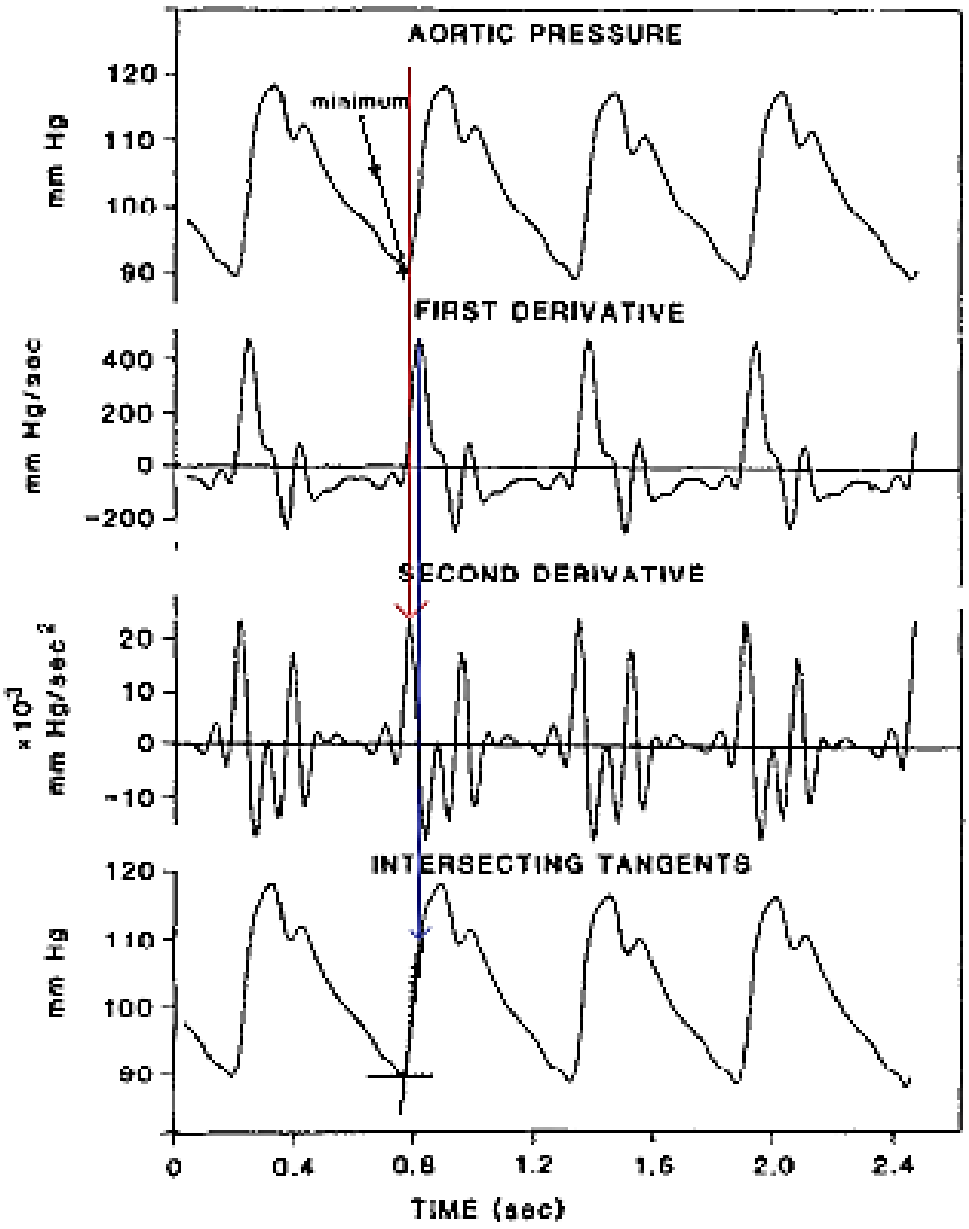

Figure 3.7. The different methods for determining the foot of the pulse pressure waveforms. The maximum point of the second derivative of the pulse pressure waveform corresponds with the maximal gradient (acceleration) at the foot of the pulse pressure wave form (depicted by the red line). The intersecting tangents method determines the foot of the pulse pressure wave as the point where a line tangent to the upstroke of the pulse pressure waveform (the line starts from the point at which the first derivative is at maximal as shown by the blue line) intersects the horizontal line at the minimum of the waveform. Modified from (Chiu *et al.*, 1991).

To determine PWV using LabChart, the tonometer signals were low-pass filtered at 50 Hz to eliminate noise and subsequently band-pass filtered

between 5-30 Hz to isolate the high frequency components of the pulse wave (i.e foot and notch of the wave) as previously described (Munakata *et al.*, 2003). The foot point of the wave was determined from the minimum point of this filtered signal (Figure 3.8). 20 clear pulse pressure waveforms from each arterial site were chosen and the time at the peak of the R-wave on the ECG and the time at the minimum point of the filtered tonometer signal were exported to an Excel spreadsheet (Microsoft Excel 2007). At each arterial site the time difference between these points from the 20 pulse pressure waves were calculated and an average PTT determined. PWV was calculated using the equation previously mentioned.

Figure 3.8. LabChart software (LabChart 7.0, ADInstruments) for recording and analysing pulse pressure waveforms for calculation of PWV. The pulse pressure waveforms captured using a tonometer are recorded in channel four. These signals are low-pass filtered at 50 Hz to eliminate noise (channel five) and subsequently band-pass filtered (5-30 Hz) to delineate the foot of the pulse wave (channel six). The minimum point of this signal is the foot of the pulse pressure waveform (depicted by the arrow).

The between-day coefficient of variation (standard deviation/average) and absolute difference for PWV_{cr} and PWV_{bf} using each of the methods was determined from 7 participants assessed on two separate days, four weeks apart (Table 3.2). The most reproducible method was the intersecting tangents method using the SphygmoCor PWV system as this gave the smallest between-day coefficient of variation and absolute difference. Therefore, in this thesis PWV was calculated using the intersecting tangents method.

Table 3.2. The between-day reproducibility for carotid to radial PWV (PWV_{cr}) and brachial to foot PWV (PWV_{bf}) using the intersecting tangents and second derivative method on the SphygmoCor PWV system and the LabChart analysis method.

Method	PWV_{cr}		PWV_{bf}	
	Coefficient of variation (%)	**Absolute difference (m/s)**	**Coefficient of variation (%)**	**Absolute difference (m/s)**
Intersecting tangents	9.5	0.6	10.0	0.2
Second derivative	10.5	0.7	10.9	0.2
LabChart	10.9	0.8	13.9	0.7

3.6.3 Validity of applanation tonometry for PWV assessment

Applanation tonometry by a pressure sensitive probe to non-invasively measure pulse pressure and pulse pressure waveforms is a validated and accurate measure of intra-arterial pressure (Kelly *et al.*, 1989b; Chen *et al.*, 1996). The intersecting tangents method is a reproducible method for calculating PWV when pulse pressure waveforms are determined invasively and non-invasively using a tonometer (Chiu *et al.*, 1991). Additionally, the SphygmoCor system and tonometer for calculating aortic PWV and PWV_{cr}, has good within and between-observer reproducibility (Wilkinson *et al.*, 1998). A strong positive correlation ($r = 0.87$) exists between PWV_{bf} and aortic PWV (Yamashina *et al.*, 2002). Additionally, PWV_{bf} shows a strong association with CVD risk factors

(Munakata *et al.*, 2003; Yamashina *et al.*, 2003; Imanishi *et al.*, 2004a). In 4112 females (+30 yrs), the optimal cut-off value of PWV_{bf} for detecting individuals with more than a moderate risk of developing CVD (based on the Framingham score) was 14 m/s, with a sensitivity and specificity of 73% and 70%, respectively (Yamashina *et al.*, 2003). Therefore PWV_{bf} is a valid measure of central arterial stiffness. Brachial artery stiffness is reflected by PWV_{cr} as a measure of conduit artery stiffness (McEleavy *et al.*, 2004; Mitchell *et al.*, 2010). Thus, PWV_{cr} is a valid measure of upper limb peripheral arterial stiffness.

3.7 Blood pressure measurement

Blood pressure was auscultated 3 times at the brachial artery using a manual or automated sphygmomanometer (outlined within each chapter). These measurements were separated by a 5 min period and the average systolic (SBP) and diastolic blood pressure (DBP) were recorded. Brachial artery mean arterial pressure (MAP) and pulse pressure (PP) were calculated using the following equations.

$$MAP = {}^{2}/_{3}\, DBP + {}^{1}/_{3}\, SBP$$

$$PP = SBP - DBP$$

3.8 Carotid arterial stiffness assessment

3.8.1 Ultrasound imaging and applanation tonometry procedure to measure carotid arterial diameters and SBP

Carotid arterial distensibility, cross-sectional compliance and far wall intima-media thickness (IMT) are variables that reflect the stiffness of the carotid artery and were measured using a combination of ultrasound imaging to determine vessel diameters, and applanation tonometery to estimate carotid artery SBP. The ultrasound probe was placed on the right common carotid artery, proximal

to the bifurcation until a clear image was obtained and the tunica media and tunica intima were visible. Two video clips were captured for duration of 20 s at a rate of 15 frames per second using vascular imaging software (Vascular Imager, Medical Imaging Applications). Immediately post recordings a tonometer (model SPT-301, Millar Instruments Inc.) was held against the left common carotid artery to capture at least 20 carotid artery pulse pressure waveforms (refer to section 3.6 for details on applanation tonometry). The tonometer signals were outputted from the SphygmoCor pulse wave velocity system (SCOR-Vx, AtCor Medical Pty Ltd) to PowerLab (Powerlab model ML, ADInstruments) and recorded on LabChart software (LabChart 7.0, ADInstruments) for analysis to determine carotid artery SBP (refer to section 3.8.3).

3.8.2 Carotid arterial diameters and end-diastole IMT analysis

The video clips were analysed using semi-automated edge detection software (Carotid Tools Analysis; Medical Imaging Applications, Coralville, Iowa, USA) to determine carotid arterial diameters and far-wall IMT (Figure 3.9). A region of interest was drawn around the area of the vessel with the most defined intima-media borders. Frames were manually edited if the incorrect border was detected. However, some frames were distorted and difficult to manually edit and were therefore rejected. The diameters and the corresponding far-wall IMTs were transferred to Microsoft Excel (Microsoft Excel 2007) where the maximum and minimum diameters from 10-20 cardiac cycles were determined. Since the point at which the carotid artery diameter is at a minimum reflects end-diastole, the corresponding IMT from the minimum diameters were determined and an average calculated across 10-20 cardiac cycles. Maximum and minimum

carotid artery cross-sectional area (CSA) was calculated as an average from the 10-20 maximum and minimum carotid artery diameters as follows:

$$CSA\ (mm^2) = \pi(\frac{d_{\max or\ min}}{2})^2$$

Where d_{max} and d_{min} are the maximum and minimum carotid arterial diameters respectively.

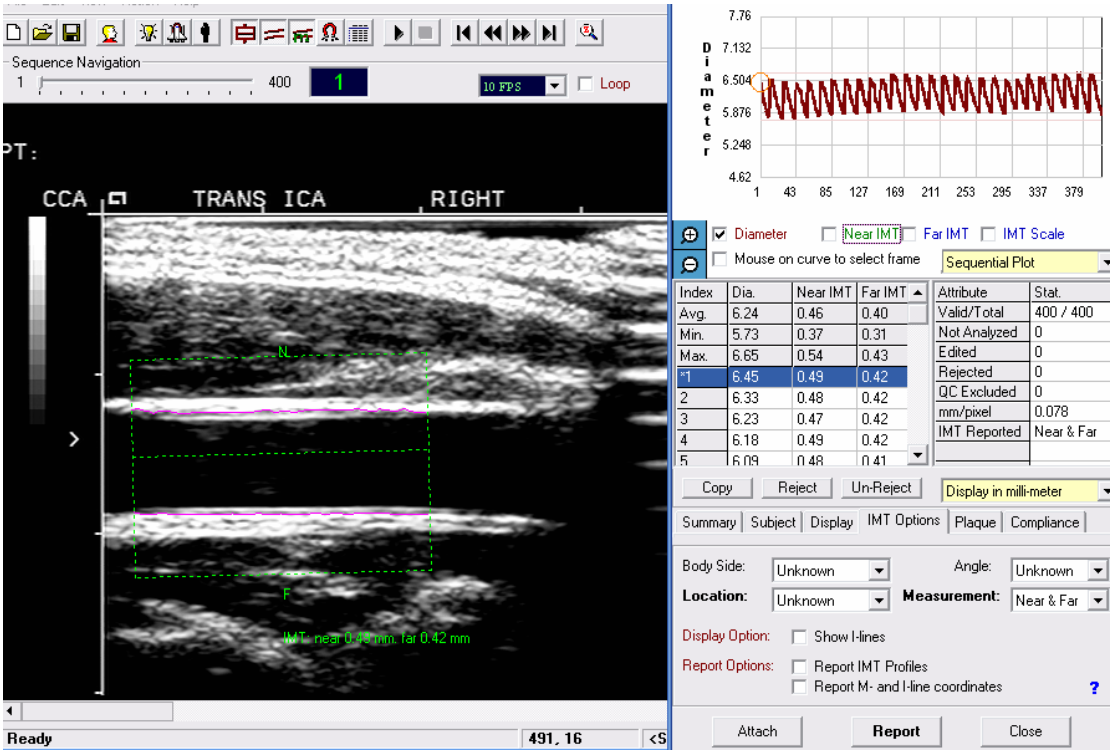

Figure 3.9. Carotid Tools analysis software for determining Carotid artery diameters and IMT.

3.8.3 Calculation of carotid arterial stiffness variables

To calculate carotid arterial stiffness, carotid artery SBP is required and can be estimated non-invasively using linear extrapolation. It was assumed that brachial artery DBP and MAP (refer to section 3.7 for the blood pressure measurement procedure) were equivalent to that in the carotid artery as differences in conduit arteries are small when in a supine position (Nichols & O'Rourke, 2005). The minimum, mean and maximum voltage values from the 20 carotid artery pulse pressure waveforms were extracted using LabChart

software (refer to section 3.8.1) and transferred to an Excel spreadsheet (Microsoft Excel 2007). Linear extrapolation involved equating the minimum and mean carotid waveform values to brachial artery DBP and MAP respectively, and used the maximum carotid waveform values as an extrapolation point to estimate carotid artery SBP, as previously described (Armentano *et al.*, 1995). The average carotid artery SBP was then calculated from the 10-20 carotid artery pulse waveforms for the calculation of carotid artery pulse pressure (PP). Carotid artery PP, cross-sectional compliance (CSC), distensibility and β-stiffness index (SI) were calculated from the following equations (O'Rourke *et al.*, 2002) :

$$PP\ (mmHg) = \ Carotid\ SBP - Brachial\ DBP$$

$$CSC\ (mm^2/mmHg) = \frac{\Delta CSA}{PP}$$

$$Distensibility\ (mm/mmHg) = \frac{CSC}{CSA_{min}}$$

$$SI\ (a.u.) = \ \ln\frac{(carotid\ SBP/DBP)}{(d_{max} - \ d_{min})/d_{min}}$$

Where PP = carotid artery pulse pressure, ΔCSA = average change in carotid artery cross-sectional area, CSA_{min} = average carotid artery minimum cross-sectional area and d_{max} and d_{min} are the maximum and minimum carotid arterial diameters respectively.

3.8.4 Reliability and validity of the assessment of carotid arterial stiffness

Carotid artery IMT and distensibility are significantly associated with CVD risk (Simons *et al.*, 1999). The use of ultrasound in combination with edge-detection software is a reproducible method for analysis of carotid artery IMT and diameters (Selzer *et al.*, 2001). Furthermore, ultrasonic measurement of carotid

IMT can be used to identify asymptomatic individuals that are at a greater risk of developing cardiovascular disease (Stein *et al.*, 2008). Greater carotid arterial distensibility is associated with lower carotid IMT and greater aortic and carotid atherosclerotic plaques (Van Popele *et al.*, 2001). Moreover, carotid artery β-SI is a measure of arterial elasticity independent of blood pressure, which increases with age (Kawasaki *et al.*, 1987) and is associated with greater severity of carotid arterial atherosclerosis (Wada *et al.*, 1994). The sensitivity and specificity for diagnosis of advanced atherosclerosis was 80% for both, when patients had a β-SI of >13 (Wada *et al.*, 1994). Thus, these measures of arterial stiffness are valid indicators of CVD risk. Validation of measuring carotid artery SBP for calculation of arterial distensibility and compliance by ultrasound and applanation tonometry is evidenced from studies adopting this same method, which have demonstrated increased carotid arterial stiffness and SBP with ageing and improvements following exercise (Tanaka *et al.*, 2000; Tanaka *et al.*, 2002). The between-day reproducibility for carotid artery IMT, CSC, distensibility and SI measured in 7 participants on 2 separate days, 4 weeks apart are presented in Table 3.3.

Table 3.3. The between-day reproducibility for carotid artery stiffness variables.

	Coefficient of variation (%)	**Absolute difference**
IMT	3.7	0.007 mm
CSC	17.4	0.01 mm^2/mmHg
Distensibility	14.8	0.0003 mm/mmHg
SI	13.2	0.18 a.u.

3.9 Blood collection procedure

A fasted venous blood sample was collected at the antecubital fossa using a syringe and a butterfly needle. Immediately after blood draw, blood was divided into EDTA vacutainers for the assessment of plasma inflammatory markers and cytokines and CAC number and function. In chapter 6, 9 ml of blood was transferred into a 10 ml tube containing 1 ml of sodium citrate for collection of platelet free plasma.

3.10 Plasma inflammatory markers and cytokines

Blood collected in EDTA vacutainers was centrifuged at 3000 g for 10 min to separate blood plasma. Blood collected in the sodium citrate tube was centrifuged at 4000 g for 10 min to separate blood plasma. The blood plasma supernatant was collected and centrifuged further at 11,000 g for 4 min to separate the platelet free plasma. The plasma supernatants were aliquoted into 1 ml volumes and frozen at -80°C until analysis. Enzyme Linked Immunoassay (ELISA) kits were used to measure plasma concentrations of Tumour Necrosis Factor- alpha (TNF-α), Interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 alpha (SDF-1α), vascular Cell Adhesion Molecule-1 (VCAM-1; R&D systems, Minneapolis, MN, USA) and high sensitivity C-Reactive Protein (hsCRP; BioVendor, Brno, Czech Republic) according to the manufacturer's instructions. For analysis of SDF-1α, platelet free plasma was analysed as its receptor CXCR4 is present on platelets and would influence the results. Standards and participant plasma samples were performed in duplicate and a microplate reader (MRX-TC II microplate reader, Dynex Technologies, Inc, Chantilly, VA, USA) used to determine the colour absorbance of each well. Analysis software (Revelations V 4.21, Dynex Technologies) was used to plot the standard calibration curve and linear line of

best fit between the optical density and concentrations of the standards, for the purpose of determining the concentration within the participants' plasma sample.

3.11 Measurement of circulating markers of endothelial damage and repair

3.11.1 Principles of flow cytometry for cell enumeration

A flow cytometer is a technology that delineates different cell populations based upon the size and granularity of the cell. In haematology the particles or types of cells within the blood (e.g. leukocytes) can be distinguished further by labelling cells with fluorochrome-conjugated antibodies that bind to specific antigens on the cell. Given that one antibody can be present on several cell types, several antibodies attached to different fluorochromes may need to be added to one sample, to distinguish between cell types (Brown & Wittwer, 2000). Cells within a sample are drawn into the flow cytometer in a single line to allow measurements to be made on an individual basis. As each cell passes through a laser light beam, light is scattered in all directions. Light that is scattered in the forward direction (forwards scatter; FSC) is detected by a sensor placed in the path of the laser beam and provides information on the size of the molecule, with a larger magnitude of scatter reflecting a larger cell. Light that is scattered from the cell at a 90° angle to the laser beam is detected by a side scatter detector (side scatter; SSC) with a greater side scatter associated with a greater granularity of the cell. When the laser beam hits a fluorochrome that is conjugated to an antibody attached to the cell, fluorescent light of a different wavelength to the excitation laser beam is emitted and detected by fluorescent sensors (Ormerod, 2000). This data is collected and analysed by computer software.

3.11.2 Enumeration of CACs

3.11.2.1 Human CAC enrichment and enumeration kit

A 21 ml blood sample was divided into two 10 ml (CAC sample and control sample CD309/VEGFR-2/KDR) and one 200 µl (control sample CD133) samples for analysis using a human CAC enumeration kit (EPC enrichment and enumeration kit, Miltenyi Biotec). Red blood cell lysis was added to all samples and gently rotated for 10 min. Following this, 20 µl of lysed blood was removed for total white blood cell enumeration by haemocytometry. All samples were centrifuged for 10 min at 300 g. The supernatants were removed and the pellets resuspended in fluorescence-activated cell-sorting buffer (FACS buffer; PBS with 0.5% bovine serum albumin and 2 mM EDTA). In the CAC and CD309 samples, 100 µl of Fc-Receptor blocking reagent and EPC enrichment cocktail were added to block non-specific cell binding and to magnetically label $CD34^+$ cells. Following a 30-min fridge incubation period, the CAC sample was labelled with fluorochrome-conjugated antibodies specific for CD34-FITC, CD133-PE, CD14-PerCP and CD309-APC and the CD309 sample labelled with CD34-FITC, CD133-PE, CD14-PerCP and Mouse IgG1 (CD309 isotype control). The CD133 sample was incubated with 20 µl of FC-receptor blocking reagent and CD34-FITC, Mouse IgG2b (CD133 isotype control) and CD14-PerCP. Subsequently, all three samples were centrifuged at 300 g for 10 min, and the pellets resuspended in FACS buffer. For magnetic-activated cell-sorting of $CD34^+$ cells, the CAC and CD309 samples were passed through a magnetic separation column (Miltenyi Biotec) and prepared for flow cytometry in FACS buffer. Finally, propidium iodide was applied to all samples to exclude dead cells before immediate transfer to a FACScalibur cytometer (Becton Dickinson) for analysis. Software (CellQuest; Becton Dickinson) and a gating strategy

recommended by the manufacturer (Miltenyi Biotec) was used for CAC enumeration. CACs were then calculated as the absolute number of $CD34^+CD309^+CD133^+$ per 10 ml. The reported between-day reproducibility for this method was 17% coefficient of variation (Rakobowchuk *et al.*, 2012).

3.11.2.2 International Society for Haematotherapy and Graft Engineering (ISHAGE) protocol

Due to the high cost, long protocol duration and a poor reported reproducibility of the above technique (Rakobowchuk *et al.*, 2012) a modified ISHAGE protocol was chosen for CAC enumeration in subsequent studies (Schmidt-Lucke *et al.*, 2010). The protocol is an established method for $CD34^+$ stem cell enumeration and identifies the $CD34^+CD45^{dim}$ population which are believed to include the late outgrowth CACs that hold characteristics of mature endothelial cells (Hur *et al.*, 2004; Timmermans *et al.*, 2007). A 1 ml peripheral blood sample was mixed with 1 ml of PBS and incubated with 20 µl of Fc-Receptor blocker (Miltenyi Biotec) for 10 min, before a 10 min incubation period with 10 µl of fluorochrome-conjugated antibodies, CD34-PE, CD45-FITC and CD309/VEGFR-2/KDR-APC (Miltenyi Biotec). The sample was lysed, centrifuged at 300 g for 10 min and the pellet resuspended in 1 ml of FACS buffer before final centrifugation at 300 g for 10 min. The remaining pellet was resuspended in 500 µl of FACS buffer and immediately enumerated by flow cytometry (LSR-Fortessa, Becton Dickinson) and CellQuest software (Becton Dickinson). Samples were analysed within 2 hrs of blood collection and at least 100,000 events recorded in gate R1 (Figure 3.10). Cells were gated using analysis software (FlowJo7 6.4) and $CD34^+$ cells defined as cells in gate R2 and $CD34^+/CD45^{dim}$ as cells in gate R5 (Figure 3.10). Cells were expressed as an absolute number per 100,000 leukocytes. Isotype controls were not used as they were not recommended. However, during analysis it became apparent that a CD309/VEGFR-2/KDR-APC isotype

control was required to enable the placement of the quadrant gate (Figure 3.11f). Additionally, a defined cluster of $CD34^+$ cells were not appearing in R2 (Figure 3.10), therefore modifications were made to the protocol for chapter 6.

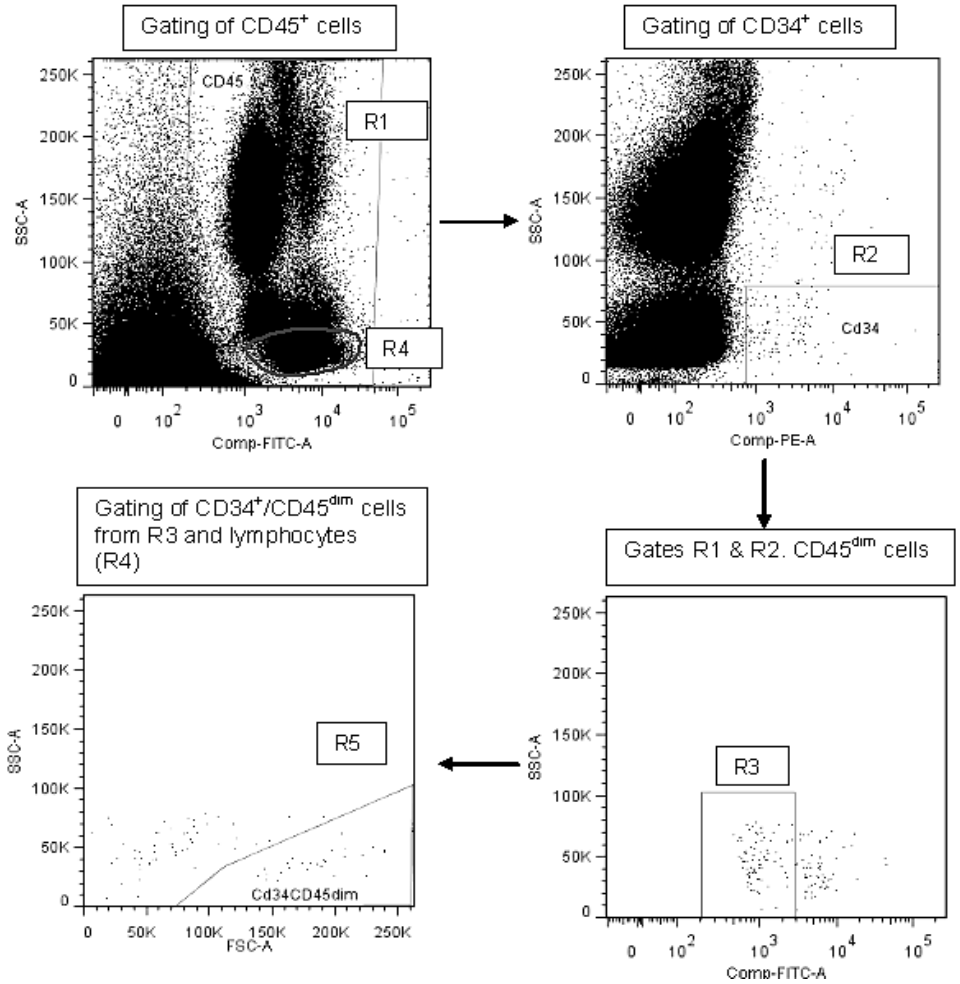

Figure 3.10. ISHAGE gating strategy for CAC enumeration. Cells positive for CD45 and therefore leukocytes were gated in R1. Cells in R1 that are positive for CD34 and have low side scatter characteristics were plotted in R2 and defined as $CD34^+$ cells. Cells from R2 were gated on a further plot to determine the $CD45^{dim}$ population. Subsequently cells from R3 were back-gated from R4 and gated on a further plot (R5) to define $CD34^+CD45^{dim}$ cells that are located within the lymphocyte population.

3.11.2.3 Modified ISHAGE protocol for inclusion of CD309/VEGFR2 CACs

A 10 ml blood sample was mixed with 40 ml of red blood cell lysis and gently rotated for a 10 min period. From the lysed blood, 20 µl was removed for a white blood cell count using a haemocytometer for calculation of the total number of white blood cells within the 50 ml lysed blood sample. 20 million cells were removed and centrifuged at 300 g for 10 min and the pellet resuspended

in 200 µl of FACS buffer. The sample was divided into two (CAC and isotype control) to give 10 million cells per sample. This was to ensure a large number of $CD34^+$ cells would be present for a cluster to appear during analysis. According to the manufacturer's guidelines (Miltenyi Biotec), each sample was incubated with 20 µl of Fc-Receptor blocker for 10 min before 10 µl of the appropriate antibodies were added for a 10 min incubation period in the fridge. In the CAC sample, cells were labelled with CD34-PE, CD45-FITC and CD309/VEGFR2/KDR-APC. In the isotype sample, cells were labelled with CD34-PE, CD45-FITC and IgG1-APC (isotype control). Unbound antibodies were washed with 1 ml of FACS buffer and centrifuged at 300 g for 10 min. The remaining pellets were resuspended in 500 µl of FACS buffer and taken to the flow cytometer (LSR-Fortessa, Becton Dickinson) for analysis. A sequential gating strategy was employed as previously described but with the addition of a CD309/VEGFR2/KDR-APC quadrant (Figure 3.11). The number of $CD34^+$ (Figure 3.11b) and $CD34^+CD45^{dim}$ (Figure 3.11e) cells were calculated as an average from the CAC and isotype samples. The number of $CD34^+CD45^{dim}KDR^+$ CACs was determined from Q2 of the CAC sample (Figure 3.11f). The between-day reproducibility for CAC number, measured in 3 participants on 2 separate days, are presented in Table 3.4.

Table 3.4. The between-day reproducibility for circulating angiogenic cell (CAC) number.

CACs	Coefficient of variation (%)	Absolute difference (/10^5 leukocytes)
$CD34^+$	9.7	4.6
$CD34^+CD45^{dim}$	15.5	0.9
$CD34^+CD45^{dim}KDR^+$	82.2	0.3

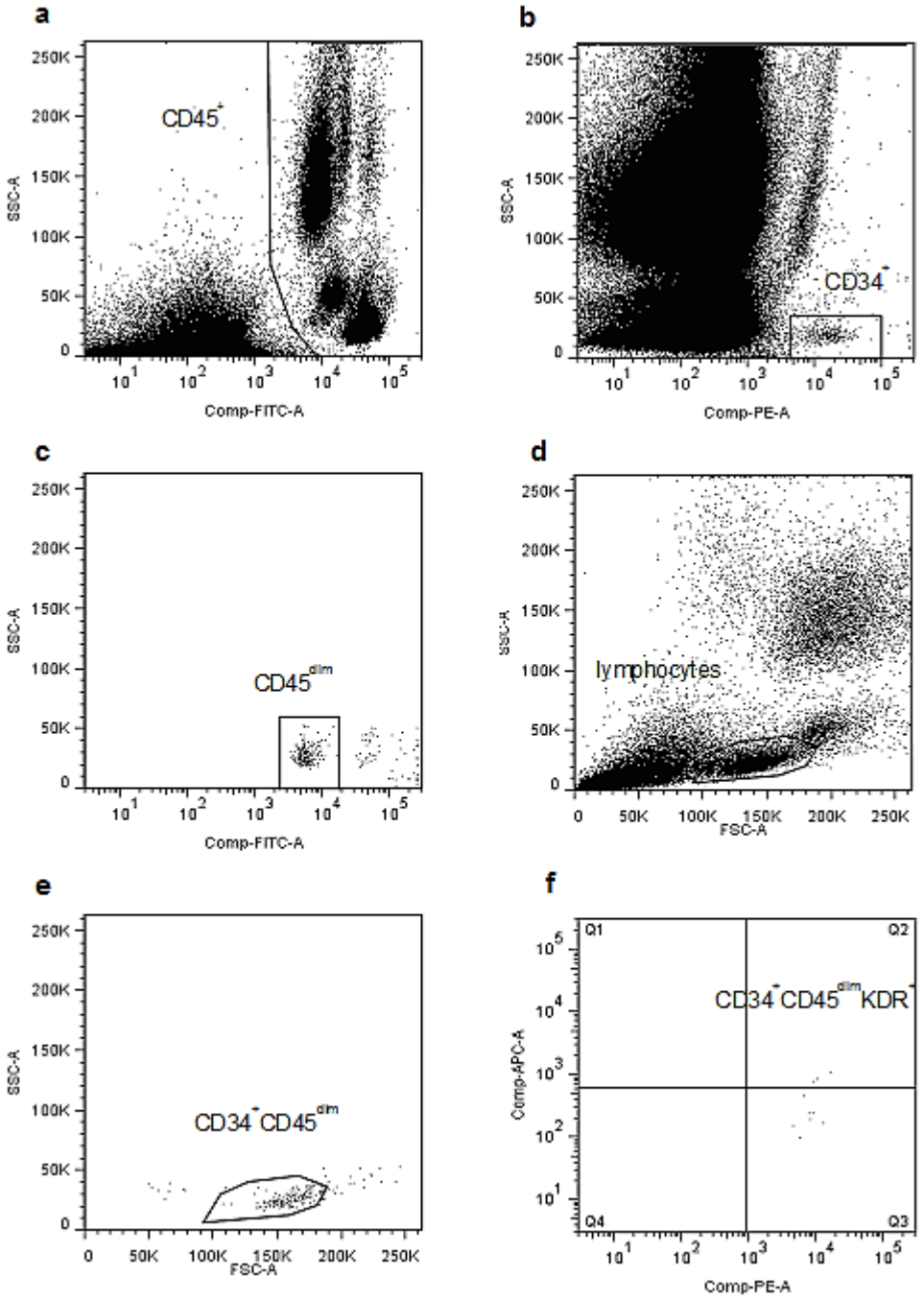

Figure 3.11. Sequential gating strategy for $CD34^{+}CD45^{dim}KDR^{+}$ CAC enumeration. Leukocytes (a) were sequentially gated to determine the presence of $CD34^{+}$ cells that have low side scatter characteristics (b). The population of $CD45^{dim}$ cells were delineated (c) and were gated further to include cells from the lymphocyte population only (d & e). Within this population the presence of CD309/VEGFR2/KDR was then determined from Q2 (f). The position of Q2 was set from the isotype sample by moving the quadrant until no cells were present in Q2.

3.12 *In vitro* assessment of CAC function

3.12.1 Culture of CACs

Peripheral blood mononuclear cells were separated from a 25-30 ml blood sample by Ficoll density-gradient centrifugation according to the manufacturer's instructions, (Ficoll Paque PLUS, GE Healthcare, Buckinghamshire, UK). The blood sample was mixed with PBS to a 50 ml volume and slowly layered on Ficoll before centrifugation at 300 g for 30 min which allowed separation of the erythrocytes, mononuclear cells and blood plasma. The middle layer containing the mononuclear cells were removed using a pipette and washed in PBS before being centrifuged at 300 g for 10 min to remove platelets, plasma and any Ficoll present in the solution. The remaining cells were suspended in endothelial growth medium (EGM; endothelial basal medium supplemented with 20% foetal calf serum, growth factors and antibiotics; EBM-2, Bullet kit; Lonza, Inc, Basel, Switzerland). Cells were plated on 6-well fibronectin coated plates (Millipore, Billerica, MA, USA) at a density of $5x10^6$ per well and cultured for 7 days at 37ºC in 5% CO_2.

To reduce costs, Millipore 6-well plates were replaced with uncoated 6-well plates (Becton Dickinson) in chapter 6. On the day prior to plating cells, 500 µl of fibronectin (20 µg/ml, Sigma-Aldrich Co Ltd, St. Louis, MO, USA) in medium 199 (M199 culture medium, Sigma-Aldrich) solution was added to each well and left overnight in the incubator. Wells were washed with PBS before cells were added. Own-coated plates were compared with the original Millipore 6-well plates to confirm fibronectin coating was working correctly. Mononuclear cells from one pilot participant were cultured in a Millipore fibronectin coated plate and one own-coated fibronectin plate. On days 2, 5 and 7 of culture, 10 images were taken in one well of each plate at 200x magnification. The number of

adherent cells per high powered field were counted and an average calculated. As shown in Figure 3.12 the number of adherent cells per high powered field per well on days 2, 5 and 7 of culture was similar between the plates with a slightly greater adherence using own-coated plates. Thus, own-coated plates were deemed appropriate for culturing CACs.

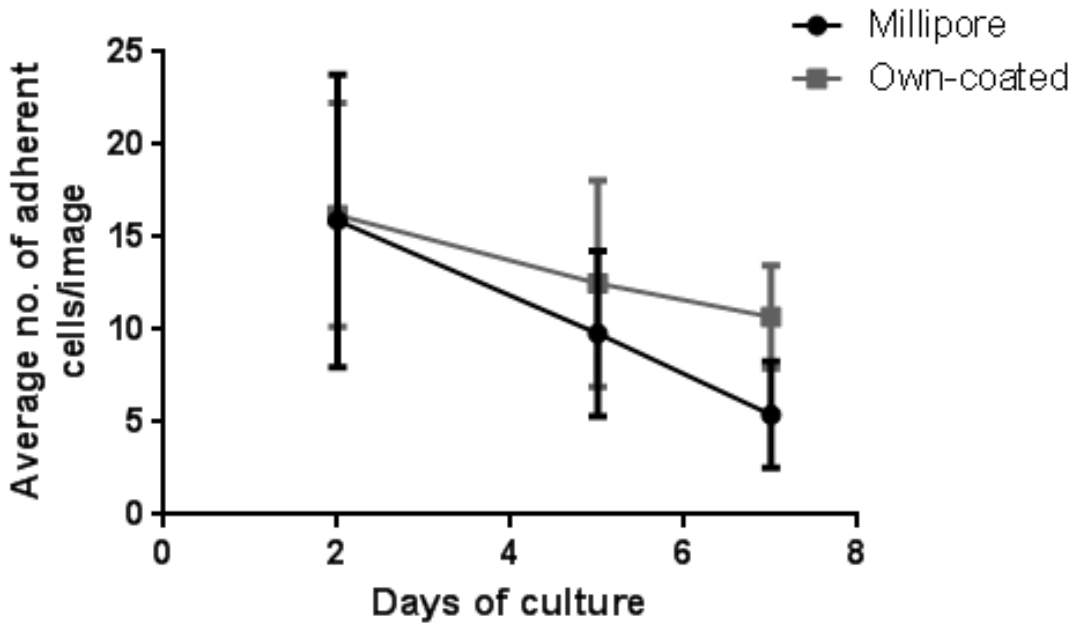

Figure 3.12. Comparison of Millipore and own-coated fibronectin plates for cell culture. The number of adherent cells (±SD) per high powered field (200x magnification) were counted on days 2, 5 and 7 of culture from one participant.

In chapter 5, wells were washed with PBS and fresh medium added daily to remove non-adherent cells. However, in subsequent studies this was modified due to the low cell numbers at harvest on day 7. In chapter 4, medium was changed on day 2, 4 and 6. In chapter 6, medium was changed on day 2 and 4 and cell culture growth was characterised (Section 3.12.1.1). The cells cultured using this method for 7 days are a heterogeneous population that migrate and adhere to areas of damage/ischaemia (Walter *et al.*, 2002) and aid in repair/angiogenesis by secreting angiogenic growth factors such as VEGF and SDF-1α (Rehman *et al.*, 2003; Hur *et al.*, 2004).

On day 7 the phenotype of the adherent CACs were measured by assessing their ability to up take acetylated DiI and lectin as previously described (Vasa *et al.*, 2001). In one well, adherent cells were washed with PBS and incubated with DiI-AcLDL (10 µg/ml in supplemented EBM-2) for two hours. Subsequently, adherent cells were fixed with 4% paraformaldehyde for 10 min followed by staining with Ulex Europaeus lectin-FITC (10 µg/ml in PBS; Biomedical Technologies Inc, Stoughton, MA, USA) and incubated for one hour in the dark. Phase contrast and fluorescent images (DiI and FITC fluorescence) were collected using an Olympus CKX-41 fluorescence microscope, and images were overlaid for quantification of cells double stained with DiI-AcLDL and lectin. In chapter 6, this staining technique was not used as it reduced the number of cells that could be used in the functional assays.

On day 7 adherent cells were detached using trypsin/EDTA solution (400 µl per well) incubated for ~90 s. Following vigorous agitation, 2 ml of EGM was added to each well to deactivate the trypsin. The cells were isolated by centrifugation at 300 g for 10 min and used in functional assessment assays (sections 3.12.3-3.12.5). To reduce intra-observer variability the same researcher conducted the functional assay analysis.

3.12.1.1 Cell culture growth characterisation

In chapter 6, cell culture growth was characterised. On day 2, 4 and 7 of culture, the non-adherent cells were removed and centrifuged at 300 g for 10 min. The cell pellet was resuspended in EGM and 20 µl removed and mixed with 20 µl of Trypan Blue (Sigma-Aldrich) for a cell count to determine the number of live and dead non-adherent cells per well using a haemocytometer. Dead cells were distinguished as dark blue cells due to the absorption of trypan blue. In one well, 10 random images (200x magnification) were taken to assess changes to

cell area during culture. From the 10 images taken on day 2, 4 and 7 of culture, 20 cells were chosen at random and cell area was quantified using analysis software (ImageJ 1.42q, Bethesda, MD, USA).

3.12.2 Colony forming unit (CFU) assay

The number of CFUs cultured *in vitro* have an inverse relationship with the presence of cardiovascular disease risk factors (Hill *et al.*, 2003). CFUs were cultured according to the manufacturer's instructions (StemCell Technologies, Vancouver, BC, Canada). The peripheral blood mononuclear cells were isolated (section 3.12.1) and $5x10^6$ cells were suspended in 2 ml of EndoCult growth medium (StemCell Technologies) and cultured in one well of a fibronectin coated 6-well plate (Becton Dickinson) for 48 hours. The non-adherent cells were collected from the well and seeded in duplicate on a 24-well fibronectin coated plate (Becton Dickinson) at a density of $1x10^6$ cells/well. Following 3 further days of culture the non-adherent cells were removed and the number of CFUs per well counted and an average calculated. CFUs were defined as clusters of >100 round cells with spindle shaped cells surrounding the core.

Due to the expense of the specialised EndoCult medium, attempts were made to culture CFUs using EGM-2 medium and M199 medium supplemented with 20% foetal calf serum (FCS), growth factors and antibiotics as originally described (Hill *et al.*, 2003). However, these attempts failed as no CFUs formed. Additionally, it has been shown that CFUs are composed mainly of monocytes/macrophages that ingest bacteria and do not form blood vessels *in vivo* (Yoder *et al.*, 2007). As the angiogenic potential of these cells are unclear and only appear in a specific medium, CFUs were not assessed in chapters 4 and 6.

3.12.3 Boyden chamber migration assay

CACs are mobilised and migrate to areas of vascular damage in response to different stimuli such as VEGF (Asahara *et al.*, 1999). The migratory ability of CACs towards VEGF can be measured *in vitro* using a Boyden chamber assay, which is impaired in the presence of cardiovascular disease risk factors (Vasa *et al.*, 2001). Boyden chambers (Becton Dickinson) containing a semi-permeable membrane with 8 µm pores were placed into wells containing either 750 µl of unsupplemented EBM (negative control) or VEGF (50 ng/ml in 750 µl EBM; R&D systems). After 7 days of culture, $4x10^4$ CACs in 500 µl of unsupplemented EBM were placed into the Boyden chambers and incubated for 24 hours. Subsequently, cells were fixed with ethanol and non-migrated cells were removed from the upper surface of the Boyden chamber with a cotton swab. Migrated cells on the lower surface of the chamber were stained with haematoxylin and eosin and 10 random microscopic images (x200 magnification) were collected for quantification of migrated cells. The ability of cells to migrate to a chemoattractant was calculated as the difference between the number of migrated cells to VEGF and the number of migrated cells to the negative control. The assay was performed in triplicate in each condition if enough CACs were harvested on day 7 and an average per 10 high powered fields calculated.

This assay was not completed in chapter 6 because the number of migrated cells was substantially lower than the reported values in the literature and in some participants CACs migrated more in the negative control. A potential reason for the low numbers could be that only $4x10^4$ CACs were used per well whereas $1x10^5$ have been used in the other studies (Vasa *et al.*, 2001).

However, this number could not be increased because not enough CACs would be available for the other functional assays.

3.12.4 CAC adhesion to fibronectin

CACs aid in vascular repair by homing and adhering to areas of damage (Urbich & Dimmeler, 2004). The adhesion to fibronectin assay has been described elsewhere (George *et al.*, 2003) and is impaired in patients with CAD (Huang *et al.*, 2007) and in-stent restenosis (George *et al.*, 2003). Thus, impaired adhesive ability of CACs may represent an impaired reparative capacity. For determination of the adhesive ability to fibronectin, CACs following 7 days of culture were placed in 24-well fibronectin coated plates (Millipore) at a density of $5x10^4$ per well in 1 ml of EGM. After incubation for 24 hrs, non-adherent cells were removed by gently washing with PBS and 10 random microscopic images (x200 magnification) were collected for quantification of adherent cells. Assays were performed in triplicate if enough CACs were harvested on day 7, and an average number of adherent cells per high powered field calculated.

3.12.5 CAC adhesion to vascular smooth muscle cells (VSMCs)

3.12.5.1 Pilot work

In chapter 6, a new method for assessing CAC function was developed as the migration assay was discontinued (see above). The adhesive ability of CACs are important for endothelial repair/angiogenesis as they act by adhering to existing endothelial cells in areas of ischaemia or vascular damage (Chavakis *et al.*, 2005) and secrete angiogenic growth factors (Rehman *et al.*, 2003). Therefore, the adhesion to fibronectin assay was modified to make it representative of endothelial damage *in vivo*. Initially human umbilical vein endothelial cells (HUVECs) were chosen instead of fibronectin as CACs from

diabetic patients with peripheral artery disease (PAD) showed impaired adhesion to HUVECs when compared to diabetic patients without PAD (Fadini *et al.*, 2006c). The assay was tested on two pilot participants. A confluent monolayer of HUVECs (Life technologies, Invitrogen) was prepared 48 hours before the assay by plating 16000 cells/cm^2 (passage 1 to 5) in wells of a 12-well tissue culture plate (Becton Dickinson). CACs from the participants were cultured for 7 days (section 3.12.1) and 10^5 adherent CACs were added to each HUVEC well and incubated at 37ºC for 4 and 24 hours. At each time point the non-adherent cells were removed by washing with PBS and 10 random microscopic images taken. However, the HUVEC monolayer changed in appearance and cells began to come off the plate (Figure 3.13). Additionally, the healthy HUVEC monolayer is not representative of a damaged vessel. CACs have been shown to adhere to the de-endothelialised area of the carotid artery following artificial injury in mice (Walter *et al.*, 2002; Werner *et al.*, 2003). Therefore, an adhesion assay was developed using vascular smooth muscle cells (VSMCs) to represent a denuded vessel.

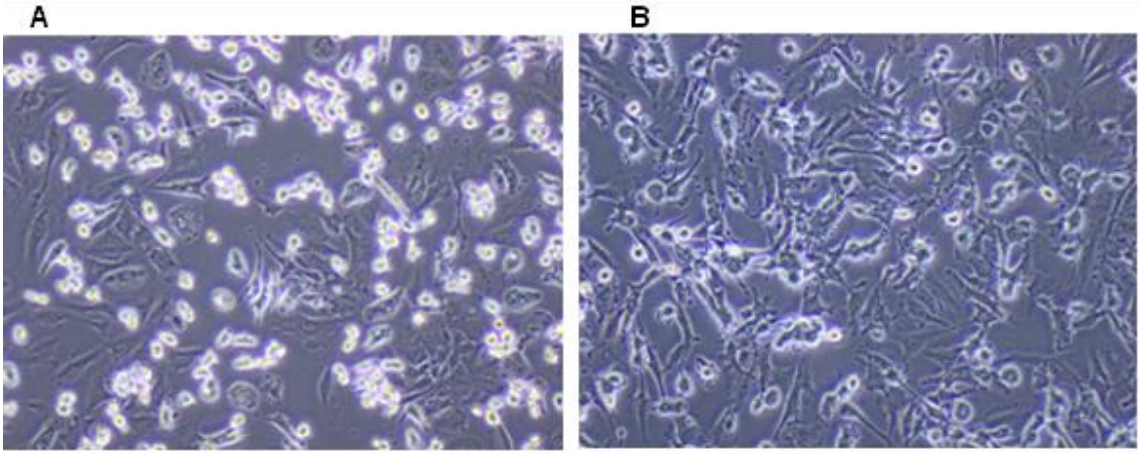

Figure 3.13. Circulating angiogenic cell (CAC) adhesion to a HUVEC monolayer after 4 hours (A) and 24 hours (B) incubation.

CACs were cultured from 2 pilot participants for 7 days and confluent monolayers of saphenous vein smooth muscles cells (passage 6) prepared by

placing $2x10^4$ cells per well of a 96-well plate. The smooth muscle cells were collected from patients undergoing coronary artery bypass graft surgery in the Leeds General Infirmary. The first pilot experiment involved determining the density of cultured CACs to apply to the VSMCs. CACs at densities of $6x10^3$, 10^4, $1.25x10^4$ and $2.5x10^4$ were added to wells of VSMCs in 250 µl of EGM. After 1 hour, non-adherent cells were removed by washing with PBS and cells were fixed with 4% paraformaldehyde (100 µl/well). 5 random microscopic images were taken per well (100x magnification) and the average number of adherent cells per image calculated. The number of adherent CACs after one hour increased with larger densities of CACs per well (Figure 3.14). Therefore, the highest density of $2.5x10^4$ was chosen so that a time course of adhesion could be measured up to one hour.

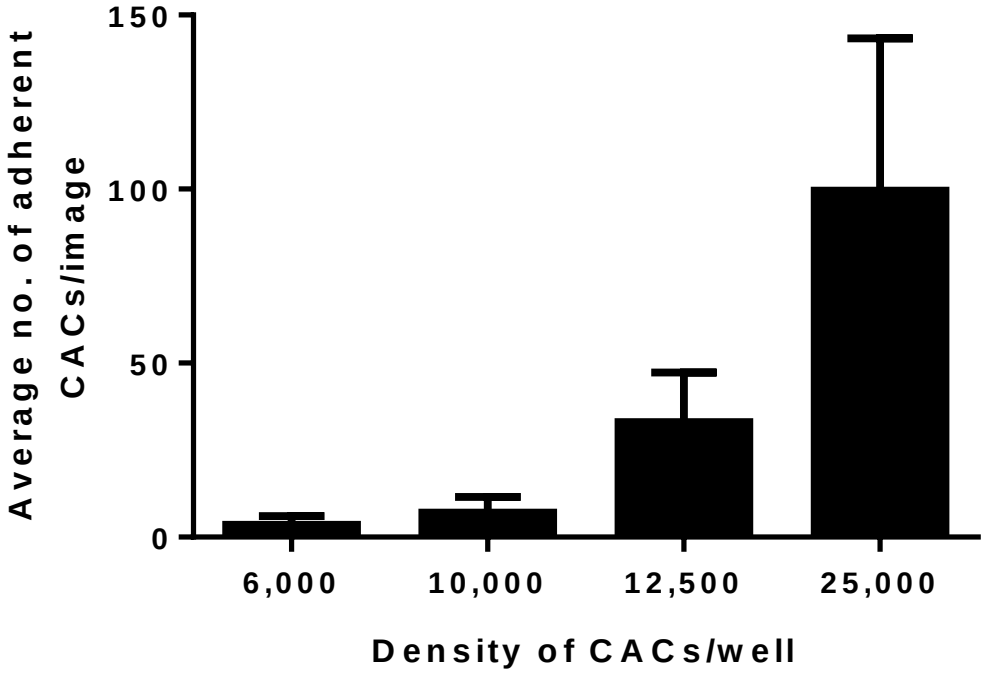

Figure 3.14. Determining the density of CACs for the adhesion to saphenous vein smooth muscle cell assay. The average number (+SD) of adherent cells per microscopic image increased with the higher the density of CACs.

Using the VSMCs from the same passage, CACs from the second pilot participant were added to the monolayers in duplicate at a density of $2.5x10^4$/ well. After 10 min, 20 min, 30 min and 60 min the non-adherent cells were removed by washing with PBS. Cells were fixed with 4% paraformaldehyde (100 µl/well) and 5 random microscopic images were taken per well (100x magnification). The number of adherent cells per image were counted and an average of the duplicate wells at each time point was calculated. The number of adherent CACs increased progressively the longer the incubation period. Unlike the HUVEC adhesion assay, the VSMCs remained as a confluent monolayer and was therefore used as a functional assay in chapter 6 (refer to section 3.12.5.2 for protocol).

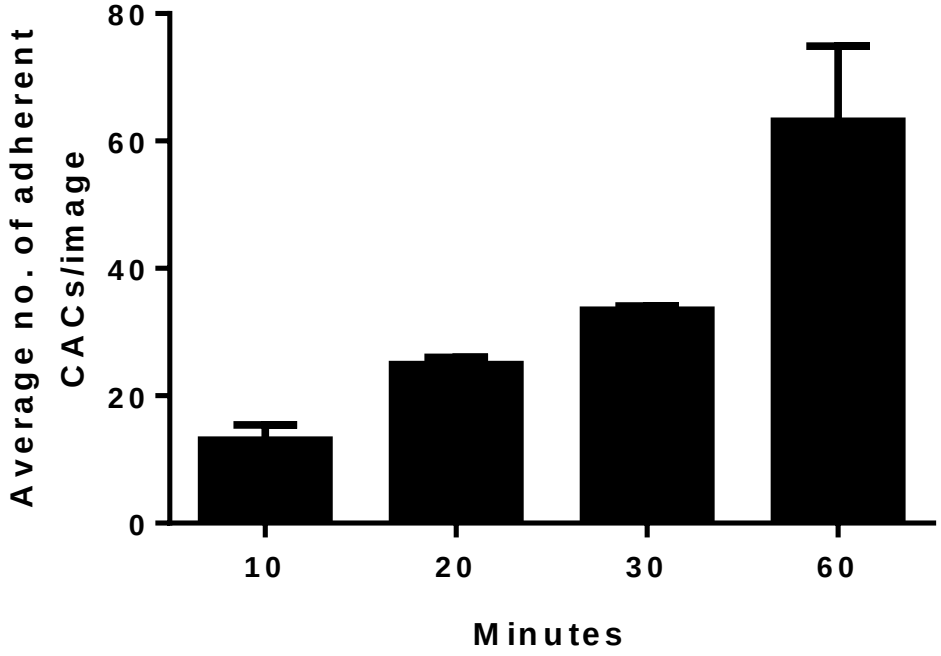

Figure 3.15. Time course of CAC adhesion to saphenous vascular smooth muscle cells. The average number (+SD) of adherent CACs were quantified following 10, 20, 30 and 60 min incubation.

3.12.5.2 VSMC adhesion protocol

Saphenous vein smooth muscle cells were collected from the Leeds General Infirmary from patients undergoing coronary artery bypass graft surgery. From the unused saphenous vein tissue, the medial layer was extracted and the smooth muscle cells were cultured and subsequently stored in liquid nitrogen until required for the adhesion assay. The cultured CACs from participants that were not being used in the paracrine function assay (section 3.12.6) were harvested on day 7 (refer to section 3.12.1). Confluent monolayers of saphenous vein smooth muscle cells (passage 2-6) were prepared in wells of a 96 well plate. CACs were placed in wells with the smooth muscle cell monolayers in duplicate at a density of $2.5x10^4$/ well in 250 µl of EGM. The number of adherent CACs to the monolayers were counted after 10, 20, 30, 60 min and 24 and 48 hrs incubation. At each time point the non-adherent CACs were removed by washing with PBS and cells were fixed with 4% paraformaldehyde (100 µl/well). Paraformaldehyde was not added at 24 hrs to allow measurement of adherence after 48 hrs. 5 random microscopic images were taken per well (100x magnification) and the number of adherent cells per image were counted and an average of the duplicate wells at each time point calculated.

3.12.6 CAC paracrine function

CACs cultured for 4-7 days secrete angiogenic growth factors and thus may aid in endothelial repair/angiogenesis in a paracrine manner through activating the proliferation of existing endothelial cells (Rehman *et al.*, 2003). To assess CACs paracrine function, condition medium was collected during culture. In detail, on day 4 of culture non-adherent cells were removed from the wells that were not being cultured for any other functional assay. The wells were washed in PBS

and 1 ml of M199 medium supplemented with 1% FCS (Sigma-Aldrich) was added to each well and incubated for 24 hours. The conditioned medium was removed on day 5 and centrifuged at 600 g for 10 min at 4°C to remove any non-adherent cells. The medium was snap frozen in liquid nitrogen and stored at -80°C until analysis. To normalise the amount of angiogenic growth factors in the medium to 10^5 CACs, the number of non-adherent cells in the pellet following conditioned medium centrifugation and the number of adherent cells in one well were counted using haemocytometry. The number of adherent cells were detached using trypsin and centrifuged at 300 g for 10 min (refer to section 3.12.1). The amount of VEGF, SDF-1α and granulocyte colony stimulating factor (G-CSF) in the conditioned medium was measured using ELISA kits according to the manufacturer's instructions (R&D systems). Standards and participant conditioned medium samples were performed in duplicate and analysed as described previously (refer to section 3.10).

3.13 Statistical analysis

All analysis was completed using statistical software (SPSS v.19, IBM Corporation, Somers, NY, USA). Data were assessed for normal distribution using the Kolmogorov-Smirnov test. If data were skewed, variables were transformed (detail is provided within the chapters) or non-parametric tests were conducted. Differences between training groups at pre-training (chapters 4 and 6) or between exercise sessions at pre-exercise (chapter 5) were assessed via a student's independent t-test and one-way ANOVA, respectively. If differences were observed, the pre-training/exercise value would be used as a covariate in the subsequent analysis. The effect of the training or exercise bout was analysed using a repeated measures ANOVA (mixed mode for chapters 4 and 6 and two-way within subject for chapter 5). Effect size was determined by

Cohen's *d* which was calculated as the difference between the pre and post-intervention means divided by the pre-intervention standard deviation of the variable (Field, 2013). The 95% confidence intervals (CIs) for the pre to post-training differences were presented in variables that showed a close to significant change following training. Pearson correlations were performed to establish relationships between variables. Significance was accepted as $p < 0.05$ and values presented as mean ± standard deviation. Power calculations were conducted using statistical software (http://hedwig.mgh.harvard.edu/sample_size/js/js_parallel_quant.html).

Chapter 4 A comparison between the effects of sprint interval and sprint continuous training on vascular health and repair in young premenopausal women

Aspects from this chapter were presented at the following conferences:

- The Systems Biology of Exercise: Cardio-respiratory and Metabolic Integration, University of Leeds, UK, August 2012.
- Annual Congress of American College of Sports Medicine, Annual Meeting, San Francisco, CA, June 2012.
- Annual Faculty of Biological Sciences Postgraduate Symposium, University of Leeds, UK, March 2012: 1st prize for institute poster presentation.

And published in:

Medicine and Science in Sports and Exercise (2012). 44(5S): S2828, p.733.

4.1 Introduction

The risk of CVD increases throughout the lifespan due in part to reduced endothelial function and nitric oxide bioavailability (Taddei *et al.*, 2001), cardio-respiratory fitness (Fitzgerald *et al.*, 1997), arterial compliance (Moreau *et al.*, 2003) and CAC number and function (Scheubel *et al.*, 2003; Heiss *et al.*, 2005). Physical activity and exercise can ameliorate these risk factors however, participation levels are low. Only 4% of women in England achieve the government recommended guidelines for exercise participation of at least 30 min of moderate-intensity continuous exercise on 5 days per week (British Heart Foundation, 2012). Furthermore, the rates of exercise participation decline throughout the female lifespan from 8% between the ages 16-34 yrs, to 0% from 65+ yrs (British Heart Foundation, 2012). Given that women reported “a

lack of leisure time" as the main barrier to exercise participation (Joint Health Surveys Unit, 2008), an increasing number of studies within the literature have focused on the benefits of high-intensity training interventions with reduced weekly time commitment. One type of high-intensity exercise emerging within the literature is sprint interval training (SIT), consisting of multiple bursts of maximal exertion separated by rest periods.

Few studies have assessed the effects of SIT upon the cardiovascular system, although Rakobowchuk *et al.,* (2008) reported an increased endothelial function and distensibility of the popliteal artery in healthy individuals after only 6 weeks of SIT. A possible mechanism for this improvement is an increased expression of endothelial nitric oxide synthase (eNOS) as observed in rodents following SIT (Laughlin *et al.*, 2004). Additionally, 6 weeks of SIT in sedentary males increased eNOS content in the muscle microvasculature in the exercising limb (Cocks *et al.*, 2013). However, the impact of SIT upon systemic endothelial function assessed at the brachial artery and on CAC number and function which aid in vascular repair, has not been investigated. Additionally, due to the active recovery periods involved in SIT sessions, total session duration is not much shorter than the recommended government guidelines of 30 min of moderate-intensity continuous exercise. A less time committing approach would be the performance of a single continuous sprint which would serve to shorten the total training session duration.

Sprint continuous training (SCT) sessions involve one sustained maximal effort sprint without rest periods. This type of training may be more appealing for training purposes than SIT as the time commitment is less. An acute bout of SCT has beneficial effects on metabolic health in overweight/obese men, with immediate increases in insulin sensitivity and reductions in insulin resistance

(Whyte *et al.*, 2013). However, the chronic effects of SCT on systemic endothelial function and repair are unknown and have not been compared with SIT. Therefore, the aim of this chapter was to determine and compare the effects of work-matched SIT with a less time committing SCT protocol on brachial artery endothelial function, arterial stiffness, cardio-respiratory fitness and CAC number and function.

4.2 Methods

4.2.1 Participants

Twelve healthy eumenorrheic females (age: 22 ± 2 yrs; BMI: 23.6 ± 1.8 $kg \cdot m^{-2}$) volunteered for the study through poster and email advertisements placed around the University of Leeds campus. Exclusion criteria other than specified in the general methods, chapter 3, section 3.1, included use of hormonal contraceptives in the last 6 months.

4.2.2 Experimental protocol

Measurements were undertaken pre and following the completion of a 4-week sprint training programme. The exercise training programme duration was chosen to ensure participants were assessed in the same phase of their individual menstrual cycle, and to determine if changes in vascular health can occur as quickly as those reported in skeletal muscle oxidative capacity (Gibala *et al.*, 2006). For both pre and post-testing assessments, participants attended the laboratory on two separate days. On the first visit the vascular measures were undertaken prior to a fasted venous blood sample collection (~31 ml) for CAC enumeration and functional assessments. On the second visit a cardio-respiratory fitness test was completed for the assessment of maximal aerobic capacity ($\dot{V}O_{2max}$) and the lactate threshold (LT). Control factors for these two sessions are specified in the general methods, chapter 3, section 3.2.

Participants were matched for relative $\dot{V}O_{2max}$ (Figure 4.2a) and assigned to either a SIT (n = 6) or SCT (n = 6) training group. Post-testing measures were acquired 4 weeks later between 48 and 72 hrs after the last training session.

4.2.3 Variables assessed pre and post-training

The protocols for the following variables are described in detail in the general methods chapter (chapter 3).

4.2.3.1 Cardio-respiratory fitness

A seated ramp incremental step exercise test (RISE-105) was performed for the assessment of $\dot{V}O_{2max}$, the LT, RI test duration and WR_{peak} (section 3.3). The ramp rate of the RI stage of the test was 1 W/4 s and the step exercise work-rate set at 105% WR_{peak}. At pre-training the SE test was excluded from analysis in 3 participants due to two participants performing non-seated cycling and equipment failure. In these participants, the $\dot{V}O_{2peak}$ value from the RI stage of the test was reported as $\dot{V}O_{2max}$.

4.2.3.2 Vascular measures

The following vascular measures were completed at pre and post-exercise training (sections 3.4-3.8): BMI, resting HR, brachial artery FMD, peak reactive hyperaemia, peak shear rate, AUC_{peak} and AUC_{60} and their corresponding VTIs, brachial artery blood pressure, PWV_{cr}, PWV_{bf} and carotid arterial blood pressure, cross-sectional compliance (CSC), distensibility, β-stiffness index and IMT. During recording of brachial artery blood velocity the Doppler insonation angle for each participant between pre and post-testing was within 3°.

4.2.3.3 CAC number and function

A 31 ml fasted blood sample was taken following the procedure outlined in section 3.9. 1 ml of the blood was analysed for CAC number via flow cytometry

following the modified ISHAGE protocol described in section 3.11.2.2. The remaining 30 ml sample was used for functional assessment of CACs. Briefly, mononuclear cells were separated from the blood, plated on fibronectin coated wells and cultured for 7 days with medium changes on days 2, 4 and 6 as described in detail in section 3.12.1. On day 7, one well of adherent CACs was stained with DiI-AcLDL and lectin to determine phenotype while the remaining wells of adherent CACs were detached and used in functional assessment assays. The ability of CACs to migrate to a chemoattractant and adhere to fibronectin were assessed (refer to sections 3.12.3-3.12.4 for details).

4.2.4 Exercise training protocol

Participants completed 3 supervised sprint training sessions per week in the laboratory for a 4-week period. All sprints were performed on a cycler ergometer (Ergomedic 874E Peak bike, Monark Exercise AB, Sweden) connected to software (Monark anaerobic test software, Monark Exercise AB HUR OY, Karleby, Finland) for the calculation of average power. In each training session participants in the SIT group completed four 30 s maximal effort sprints (Wingate test) at a resistance equivalent to 7.5% of body weight. The resistance was applied when the participant reached a cadence of 140-150 rpm and each Wingate test was separated by 4.5 min of unloaded pedalling. SIT sessions were based on previous studies (Rakobowchuk *et al.*, 2008) whereby vascular function was seen to improve with SIT training. Participants in the SCT group completed a full SIT session as their first training session for the purpose of calculating the total work achieved from the four 30 s sprints. Total work for the training session was calculated in kJ as the sum of the work in each of the 4 Wingate tests from the recording of average power using the following equation:

$$Work\ (kJ)\ = \frac{ave\ power\ x\ 30}{1000}$$

Where $ave\ power$ is the average power produced during 1 Wingate test (J/s), and $30\,s$ is the duration of the test.

The total number of revolutions performed on the cycle ergometer to achieve this total work was also calculated for the purpose of work-matching (see below) using the following equation:

$$Revolutions = \frac{work\ (kJ) / \ 0.009804}{6R}$$

Where 0.009804 is the conversion factor for kJ to kg-m, 6 is the circumference (m) of the flywheel on the cycle ergometer and R is the resistance applied (kg).

For the remaining training sessions the SCT group completed a single continuous maximal effort sprint. To enable a sustained sprint the cycle ergometer resistance was reduced by a third of the SIT session resistance to 5% of body mass. The resistance was applied when the participant reached a cadence of 140-150 rpm. To ensure the sprint was work-matched with the SIT session, the sprint was stopped when the participant achieved the equivalent total number of revolutions attained across the 4 Wingate tests in their first SIT session. As the resistance was reduced by one third, the total number of revolutions was increased by one third. Figure 4.1 shows a schematic representation of the SIT and SCT sessions. In both groups' sessions, strong verbal motivation was provided to encourage participants to maintain a fast cadence throughout the sprints. All sessions were followed by a short cool-down that was similar between the groups. Thus, the training groups by design both involved maximal exertion sprints, were matched for relative work but differed in regards to session duration and the interval vs. continuous nature of the exercise. Participants in the SCT group completed an extra 30 s Wingate

test at the end of training to assess changes in peak power from pre to post-training.

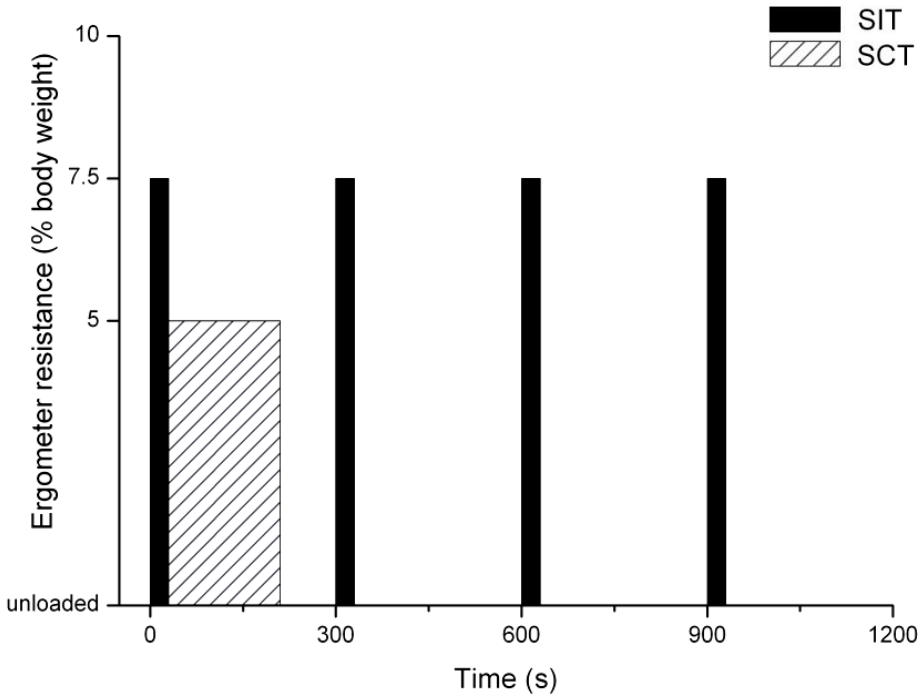

Figure 4.1. Schematic of sprint interval (SIT) and sprint continuous training (SCT). SIT sessions (solid black bars) involved a maximal 30 s sprint at 7.5% of body weight (ave: 5.1 ± 0.6 kg) followed by 4.5 min of unloaded pedalling, repeated 4 times. SCT sessions (striped gray bar) involved a maximal sprint at 5% of body weight (ave: 3.4 ± 0.4 kg) which ended when the participant completed the same amount of work which was achieved in their first SIT session. On average SCT duration was 3.5 ± 0.2 min.

4.2.5 Statistical analysis

Statistical analysis procedures are detailed in the general methods, chapter 3, section 3.13. All data were normally distributed and no training group differences in any variable were observed at pre-training. Work completed during session one was evaluated for training group differences via a Student's independent t-test. The effect of the training interventions was analysed using a mixed mode repeated measures ANOVA with time (pre vs. post-training) as the within-subjects factor and training group (SIT vs. SCT) as the between-subjects

factor. Paired t-tests were conducted for post-hoc analysis to identify which exercise bout showed a significant change. Given the lack of knowledge regarding CAC and exercise in females, CAC number was selected as the primary outcome. Using the previously reported increase of 8.7 $CD34^+$ cells/µl with a standard deviation of 3.0, following a supramaximal bout of exercise in a healthy population (Morici *et al.*, 2005), a minimum of 8 participants in total were required to obtain 80% power ($\alpha = 0.05$), if the difference between treatments was 8.7, in a two-treatment parallel-design study.

4.3 Results

4.3.1 Participants and training effect

Participant characteristics are displayed in Table 4.1. BMI, resting HR and brachial artery BP did not change following training ($p > 0.05$). By design both the training groups' session duration (SIT: 20 min, SCT: 3.5 ± 0.2 min) and resistance applied to the cycle ergometer (SIT: 5.1 ± 0.6 kg, SCT: 3.4 ± 0.4 kg) were greater in the SIT group. Work completed per session did not significantly differ between the groups (SIT: 45.9 ± 6.3 kJ vs. SCT: 47.6 ± 6.2 kJ, $p = 0.66$). Peak and average power calculated for a single 30 s Wingate test did not increase from session 1 to post-training in either group ($p > 0.05$). Both absolute ($p = 0.048$) and relative $\dot{V}O_{2max}$ ($p = 0.046$) increased with training with no time by group interaction ($p > 0.05$, Table 4.1, Figure 4.2a). The estimated LT also increased with training in both groups (Figure 4.2b); although significance was not reached ($p = 0.08$) the 95% confidence interval (CI) for pre to post-training difference ranged from -11 to 164 ml/min. The RI test duration and WR_{peak} significantly increased following training in both groups ($p < 0.001$, time by group interaction for both $p = 0.05$), with a larger increase following SIT (Table 4.1, Figure 4.2c).

Table 4.1. Participant characteristics and cardio-respiratory fitness (mean ± SD) at pre and post 4 weeks of either sprint interval (SIT) or sprint continuous training (SCT).

	SIT (n = 6)		SCT (n = 6)	
	Pre	**Post**	**Pre**	**Post**
BMI ($kg \cdot m^{-2}$)	23.6 ± 1.8	23.8 ± 1.6	23.1 ± 2.3	22.9 ± 2.6
Resting HR (bpm)	55 ± 10	56 ± 6	62 ± 8	64 ± 12
Brachial artery SBP (mmHg)	115 ± 7.0	117 ± 11	112 ± 12	111 ± 11
Brachial artery DBP (mmHg)	73 ± 5	72 ± 11	76 ± 9	72 ± 7
Brachial artery MAP (mmHg)	87 ± 5	87 ± 10	88 ± 10	85 ± 8
Brachial artery PP (mmHg)	41 ± 6	45 ± 8	36 ± 5	39 ± 7
***** Absolute $\dot{V}O_{2max}$ ($L \cdot min^{-1}$)	2.34 ± 0.37	2.55 ± 0.31	2.24 ± 0.22	2.30 ± 0.21
***** RI test duration (min)	12.14 ± 1.74	13.44 ± 1.55	12.06 ± 1.18	12.73 ± 0.9
Lactate threshold (%)	48.4 ± 7.4	46.4 ± 5.4	49.0 ± 6.4	51.9 ± 8.1

* indicates a significant main time effect ($p < 0.05$). No group differences at baseline were observed ($p > 0.05$). RI test duration time by group interaction was close to significant ($p = 0.05$) with a large effect size with SIT (SIT: $d = 0.8$, SCT: $d = 0.5$). HR = heart rate, bpm = beats per minute, SBP = systolic blood pressure, DBP = diastolic blood pressure, MAP = mean arterial pressure, PP = pulse pressure, $\dot{V}O_{2max}$ = maximal oxygen uptake, RI = ramp incremental.

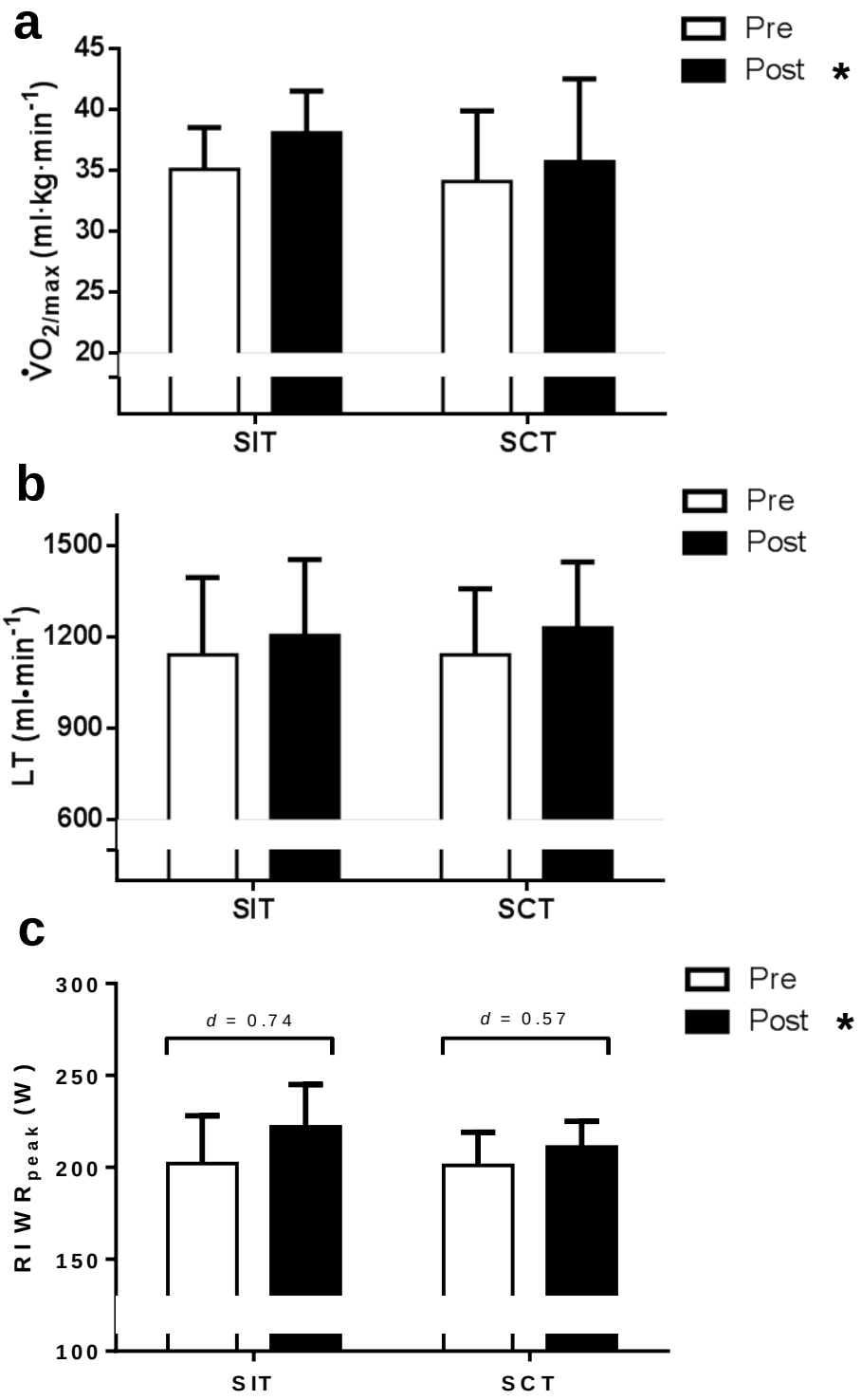

Figure 4.2. Changes in cardio-respiratory fitness following sprint interval (SIT) and sprint continuous training (SCT). * indicates a significant pre to post-training difference in both groups (time effect: $p < 0.05$). a) Relative maximal oxygen uptake ($\dot{V}O_{2max}$) increased following both SIT and SCT ($p = 0.046$) with no group by time interaction ($p = 0.49$). b) The estimated lactate threshold (LT) followed a trend to increase in both training groups (main time effect $p = 0.08$) with no group by time interaction ($p = 0.30$). c) The ramp incremental (RI) test work-rate peak (WR_{peak}) significantly increased in both groups (main time effect $p = 0.0001$) with a greater increase following SIT (group by time interaction $p = 0.05$). d = cohen's effect size.

4.3.2 Brachial artery endothelial function

Resting brachial artery diameter and time from cuff release to peak dilation were unchanged following training in both groups (p > 0.05, Table 4.2). However, absolute and relative FMD showed a trend for an increase (FMD_{rel} by ~19%, FMD_{abs} by ~23%) following SIT but little change following SCT (Table 4.2, Figure 4.3). Although the main time effect was not significant (p = 0.81), the time by group interaction showed a trend (p = 0.08), with 67% of participants in the SIT increasing FMD (FMD_{rel}: 95% CI for pre to post-training difference: -0.59 to 2.43%) and 67% of SCT participants exhibiting little change (FMD_{rel}: 95% CI for pre to post-training difference: -2.29 to 0.86%). Larger increases in absolute FMD occurred in participants with lower pre-training levels of absolute FMD (r = -0.57, p = 0.06). Peak reactive hyperaemia, peak shear rate, AUC_{peak} and AUC_{60} did not change in either training groups (p > 0.05, Table 4.2). Absolute FMD did not significantly correlate with shear rate (p > 0.05), therefore, FMD was not normalised to shear rate AUC.

Table 4.2. Brachial artery endothelial function (mean ± SD) at pre and post 4 weeks of either sprint interval (SIT) or sprint continuous training (SCT).

	SIT (n = 6)		SCT (n = 6)	
	Pre	**Post**	**Pre**	**Post**
Peak reactive hyperaemia ($cm \cdot s^{-1}$)	98.8 ± 23.6	97.3 ± 19.4	82.0 ± 14.7	90.1 ± 8.7
Peak shear rate (s^{-1})	2628.6 ± 1063.6	2564.3 ± 803.3	2126.1 ± 449.2	2394.5 ± 301.0
AUC_{peak} (a.u.)	31325 ± 11174	30406 ± 13875	27646 ± 6595	31396 ± 6366
AUC_{60} (a.u.)	39815 ± 14654	39648 ± 16146	39596 ± 5261	43901 ± 6012
Insonation angle (°)	69 ± 1	68 ± 2	68 ± 2	68 ± 1
Brachial artery baseline diameter (mm)	3.2 ± 0.8	3.2 ± 0.8	3.1 ± 0.5	3.1 ± 0.5
Absolute FMD (mm)	0.15 ± 0.09	0.18 ± 0.07	0.22 ± 0.08	0.20 ± 0.08
Time from cuff release to peak diameter (s)	38 ± 8	35 ± 4	34 ± 9	34 ± 6

Absolute FMD time by group interaction was close to significant (p = 0.08). AUC = area under the shear rate curve and FMD = flow-mediated dilation.

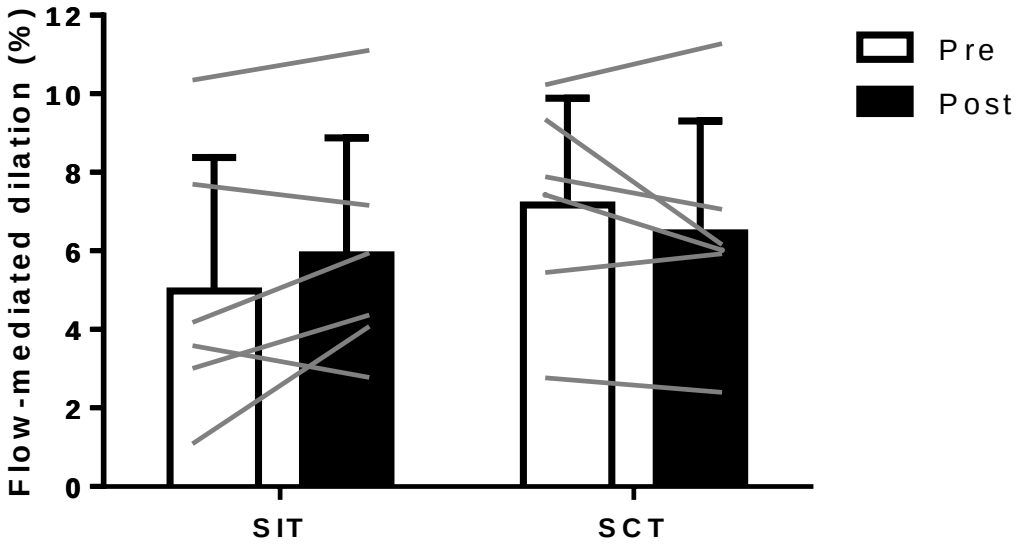

Figure 4.3. Brachial artery endothelial function following sprint interval (SIT) and sprint continuous training (SCT). Brachial artery FMD displayed a trend for an increase following SIT (n = 6) but little change following SCT (n = 6; main time effect p = 0.81; time by group interaction p = 0.08).

4.3.3 Arterial stiffness

Carotid to radial and brachial to foot PWV did not alter following training in either group ($p > 0.05$) indicating that sprint training did not affect upper limb or central PWV (Table 4.3). Carotid artery IMT, distensibility, cross-sectional compliance and stiffness index were also unaltered following both types of training ($p > 0.05$; Table 4.3).

Table 4.3. Arterial stiffness (mean ± SD) pre and post either sprint interval (SIT) or sprint continuous training (SCT).

	SIT (n = 6)		SCT (n = 6)	
	Pre	**Post**	**Pre**	**Post**
PWV_{cr} ($m \cdot s^{-1}$)	6.0 ± 0.8	6.2 ± 0.5	6.6 ± 0.8	7.4 ± 0.7
PWV_{bf} ($m \cdot s^{-1}$)	7.4 ± 0.9	7.8 ± 1.4	8.2 ± 1.6	7.4 ± 1.1
Carotid artery IMT (mm)	0.31 ± 0.10	0.36 ± 0.07	0.33 ± 0.09	0.35 ± 0.06
Carotid artery SBP (mmHg)	103 ± 6	104 ± 11	101 ± 11	98 ± 10
Carotid artery PP (mmHg)	29 ± 4	31 ± 5	25 ± 3	27 ± 5
Carotid ΔCSA within heart cycle (mm^2)	6.2 ± 1.3	6.0 ± 0.7	6.2 ± 1.5	6.2 ± 1.5
Carotid artery CSC (mm^2/mmHg)	0.22 ± 0.05	0.19 ± 0.03	0.25 ± 0.06	0.24 ± 0.08
Carotid artery DD (mm/mmHg)	0.01 ± 0.002	0.01 ± 0.002	0.01 ± 0.002	0.01 ± 0.002
Carotid artery SI (a.u.)	3.3 ± 0.8	3.6 ± 0.8	3.0 ± 0.6	3.4 ± 1.2

No group differences at pre-training, training effects or time by group interactions were found ($p > 0.05$). PWV_{cr} = carotid-radial pulse wave velocity, PWV_{bf} = brachial-foot pulse wave velocity, IMT = intima-media thickness, SBP = systolic blood pressure, PP = pulse pressure, CSA = cross-sectional area, CSC = cross-sectional compliance, DD = distensibilty, SI = β-stiffness index.

4.3.4 Training effect on CAC number

At post-testing, one participant was excluded from analysis from the SCT group due to technical problems with the flow cytometer. Circulating $CD34^+$ cells increased following both SIT (by ~44%; n = 6) and SCT (by ~28%; n = 5; main time effect $p = 0.02$) with no time by group interaction ($p = 0.83$; Figure 4.4a). However, $CD34^+CD45^{dim}$ did not change following either type of training (main

time effect p = 0.21, time by group interaction p = 0.67; Figure 4.4b). Changes in $CD34^+$ cells with training did not correlate with pre-training FMD or changes in FMD following training (Pre FMD r = 0.14, ΔFMD r = 0.34, $p > 0.05$).

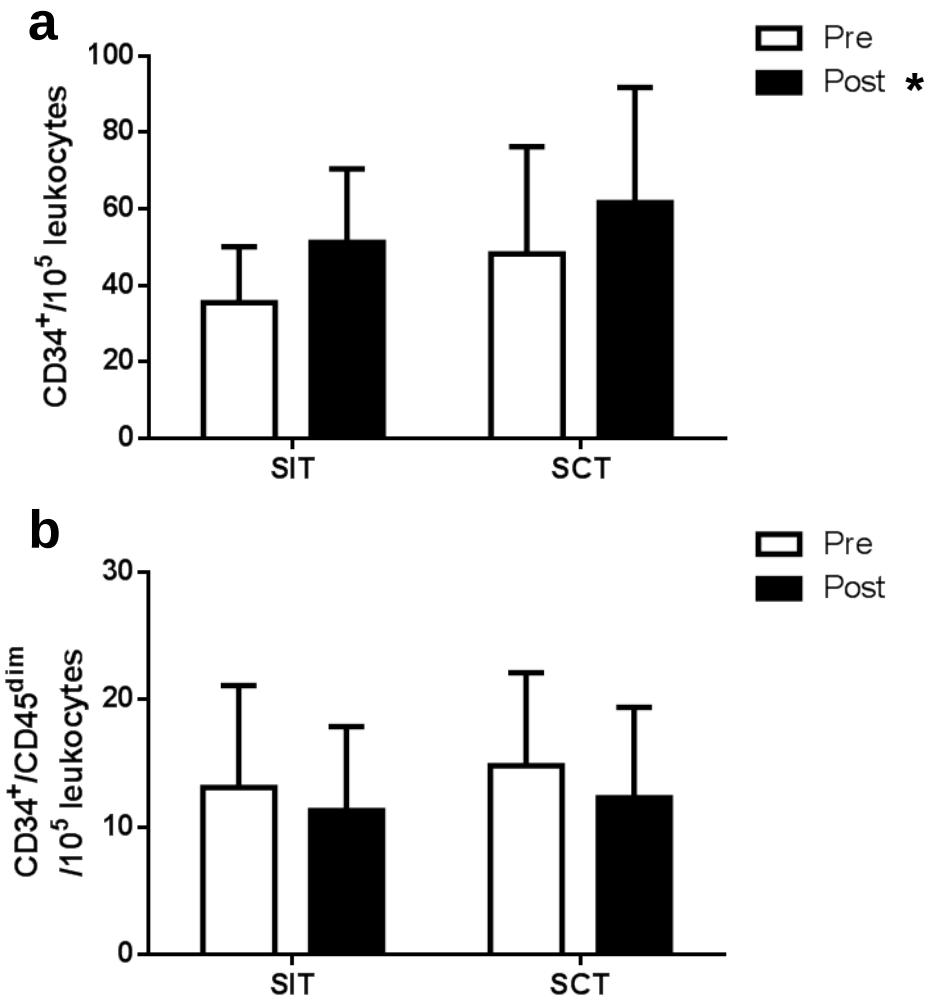

Figure 4.4. The effects of sprint interval (SIT) and sprint continuous training (SCT) on circulating angiogenic cell (CAC) number. * indicates a significant pre to post-training difference in both groups ($p < 0.05$). a) $CD34^+$ cells increased following both SIT (n = 6) and SCT (n = 5; main time effect $p = 0.02$) with no time by group interaction ($p = 0.83$). However, b) $CD34^+/CD45^{dim}$ cells did not change following either training (main time effect $p = 0.21$; time by group interaction $p = 0.67$).

4.3.5 CAC function

At post-testing 1 participant from the SCT group was excluded from the adhesion and migration assays, and 1 participant from the SIT group excluded from the migration assay due to low cell number at harvest on day 7. The

function of CACs did not significantly improve with either type of training (main time effect, adhesion $p = 0.47$, migration $p = 0.63$, DiI-AcLDL & lectin $p = 0.25$; time by group interaction $p > 0.05$; Table 4.4). However, whilst training induced changes in CAC adhesion were not related to pre-training levels ($p > 0.05$), changes in CAC migration were greater in participants with lower pre-training levels of CAC migration ($r = -0.67$, $p = 0.03$).

Table 4.4. Circulating angiogenic cell (CAC) function following either sprint interval (SIT) or sprint continuous training (SCT).

	SIT (mean ± SD)		SCT (mean ± SD)	
	Pre	**Post**	**Pre**	**Post**
DiI-AcLDL & lectin CACs/ microscopic image (SIT: n = 6; SCT: n = 6)	18 ± 7	14 ± 8	12 ± 11	11 ± 6
CAC adhesion/ microscopic image (SIT: n = 6; SCT: n = 5)	11 ± 8	8 ± 6	13 ± 8	11 ± 12
CAC migration/10 microscopic images (SIT: n = 5; SCT: n = 5)	2 ± 3	4 ± 7	1 ± 4	2 ± 2

No group differences at pre-training, training effects or time by group interactions were found ($p > 0.05$). CAC = circulating angiogenic cells.

4.4 Discussion

The present study to the author's knowledge was the first to examine the effects of sprint training on brachial artery function as an indicator of systemic vascular function, and the mobilisation and function of circulating cells that may contribute to endothelial repair. Furthermore, novel comparisons were made between SIT and SCT to explore whether sprint training involving a continuous work-rate stimulus has differential effects on the vasculature than work matched sprinting of an interval nature. The main findings were that despite the lower training time commitment, SCT improved cardio-respiratory fitness to a similar extent as SIT. Furthermore, increased mobilisation of circulating $CD34^+$ cells was observed following both types of training, but arterial stiffness,

$CD34^{+}CD45^{dim}$ mobilisation and CAC function remained unchanged. Additionally, there was a trend for brachial artery FMD to increase following SIT with little change following SCT.

4.4.1 Improvements in exercise tolerance

Recently, a “lack of time” has been highlighted as the general publics’ main barrier to exercise thus, several studies have focused on how supramaximal but less time committing exercise can be equally as beneficial to health and fitness as longer methods of exercise training. Improvements in $\dot{V}O_{2max}$ by ~10% have been observed following as little as 6 weeks of Wingate test based SIT (Burgomaster *et al.*, 2008). In agreement, the present study found similar increases in $\dot{V}O_{2max}$. Increases in $\dot{V}O_{2max}$ following exercise training involves a greater delivery and utilisation of oxygen to working muscles. From a vascular perspective, adaptations enabling this increase include capillary proliferation and an increased endothelial vasodilation and reduced arterial stiffness of the arteries supplying blood to the working muscles (Poole et al., 2012), which has been reported in the popliteal artery following SIT (Rakobowchuk et al., 2008). Other reported mechanisms for these rapid improvements include an increased skeletal muscle glycolytic and oxidative maximal enzyme activity (MacDougall et al., 1998; Burgomaster et al., 2008). Additionally, following both training types, there was a near significant improvement in the LT and a significant increase in the RI test duration and WR_{peak}, with greater improvements following SIT. Since $\dot{V}O_{2max}$ is a strong predictor of future cardiac events (Laukkanen *et al.*, 2004), and the LT and WR_{peak} are markers of exercise tolerance that are associated with poorer cardiac outcomes (Myers *et al.*, 1998), this type of training may be favourable to those who wish to rapidly increase their cardio-respiratory fitness and/or cardiovascular health. Thus, participation in this type

of exercise in women throughout the lifespan, may partly mitigate the decline in cardiovascular health and fitness associated with ageing and menopausal status.

4.4.2 Differential effects of SIT and SCT on brachial artery endothelial function

Although the sample size is small, the results indicated a close to significant trend for an increase in brachial artery FMD following SIT but little change after SCT, without any changes in peak reactive hyperaemia, peak shear rate, AUC_{peak} or AUC_{60}. Several reasons may explain this potential difference between SIT and SCT. Given that brachial artery FMD is largely nitric oxide dependent (Doshi *et al.*, 2001), this result suggests that SIT may provide a greater stimuli for increased nitric oxide bioavailability than SCT. Improvements in brachial artery endothelial function following lower limb exercise training in healthy populations have occurred as early as 2 weeks and begun to return to baseline at 4 weeks due to arterial remodelling (Birk *et al.*, 2012). This may explain why other studies adopting longer training protocols have not observed a change (Rakobowchuk *et al.*, 2012). Therefore, it is plausible that if endothelial function in the present study had been assessed after 2 weeks a larger magnitude of change may have been present.

Increases in brachial artery FMD following exercise training are believed to be caused by increases in brachial artery blood flow antegrade shear stress (Tinken *et al.*, 2010) which induce nitric oxide release through activation of eNOS (Boo *et al.*, 2002; Hambrecht *et al.*, 2003). However, in the initial 5 min from lower limb cycling onset, mean brachial artery blood flow decreases due to an increase in retrograde flow caused by forearm resistance vessel vasoconstriction as shown in Figure 4.5 (Thijssen *et al.*, 2009a; Simmons *et al.*,

2011). As opposed to antegrade shear stress, retrograde shear stress is pro-atherogenic as it increases reactive oxygen species, which reduces nitric oxide bioavailability (Laughlin *et al.*, 2008). Additionally, greater oscillatory and retrograde shear rate in the brachial artery, activates the endothelium and increases damage, evidenced by significantly higher levels of circulating endothelial microparticles during the period of induced disturbed flow (Jenkins *et al.*, 2013). Furthermore, retrograde shear stress reduces brachial artery FMD in a dose-dependent manner (Thijssen *et al.*, 2009c).

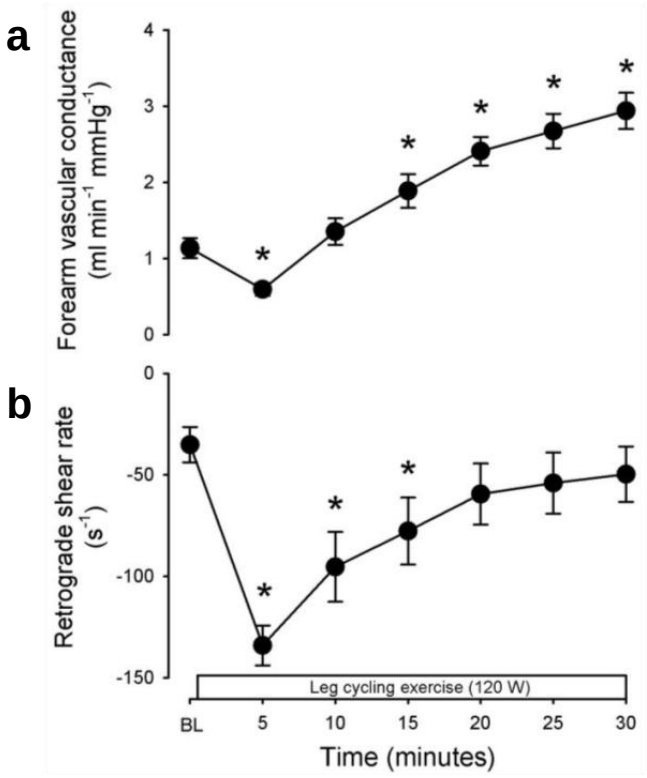

Figure 4.5. Upper arm blood flow response to lower limb cycling exercise. Forearm vascular conductance (a) and brachial artery retrograde shear rate (b) during 30 min of lower-limb cycling exercise at 120 W in 14 young healthy male participants. Reproduced from Simmons *et al*., (2011).
*** indicates a significant difference from baseline ($p < 0.05$).**

Since SCT duration was less than 5 min in our study, it is possible that the brachial artery endothelium experienced retrograde shear stress, which may have negated improvements in endothelial function by reducing nitric oxide bioavailability. In contrast, SIT duration was 20 min which allows sufficient time

for antegrade shear stress to increase and for retrograde shear stress to return to baseline due to reductions in downstream peripheral resistance caused by thermoregulatory cutaneous vasodilation (Simmons *et al.*, 2011). Thus, SIT with intervals of 30 s may be more beneficial for brachial artery adaptations due to increases in antegrade shear stress that augment nitric oxide production, whereas SCT duration may be too short to induce the increases in antegrade shear stress required for improvements in FMD. However, further studies investigating blood flow responses to SIT and SCT are required to validate this suggestion.

Superior health benefits have been reported following interval exercise training compared to continuous exercise training, which have often been related to a higher exercise intensity, shear stress and work done experienced during interval exercise (Wisloff *et al.*, 2007). In the present study, SCT and SIT were both of a high intensity and matched for work. Therefore, it may be that the profile of the repeated increments and decrements in work rate provided a greater stimulus for vascular adaptations than a continuous work rate. Evidence from *in vitro* studies on endothelial cells suggests that the temporal gradients in shear stress are more important than the magnitude of the shear for eNOS activation and nitric oxide production. A rapid increase in shear stress from a pre-existing level creates a burst in nitric oxide production (Kuchan & Frangos, 1994). Subsequently, exposure to a sustained magnitude of this shear stress reduced the rate of nitric oxide production. Additionally, repeated impulses in shear stress have been seen to produce a large increase in nitric oxide production, whereas a slow ramp increase in flow to the equivalent magnitude had no effect (Dusserre *et al.*, 2004). This was thought to be due to the activation of eNOS by platelet endothelial cell activation molecule-1 (PECAM-1)

which may only sense shear stress when rapid changes occur due to its sheltered position at cell-cell junctions (Dusserre *et al.*, 2004). Therefore, given that our SIT sessions involved 4 periods of rapid increases in shear stress compared to a single increase in the SCT, it seems reasonable to suggest that SIT provided a greater stimulus for nitric oxide synthesis. Furthermore, it is likely that the elevation in shear stress would have occurred for longer during SIT due to longer session duration. The measurement of shear stress during SIT and SCT and further *in vitro* studies examining endothelial cell responses to continuous and periodic fluctuations in shear stress are required to validate these suggestions.

4.4.3 Unaltered arterial stiffness following sprint training

PWV, a measure of arterial stiffness associated with increased CVD risk (Nichols & O'Rourke, 2005), did not change with training both centrally and in the upper limb, indicating an unaltered arterial stiffness. Conversely, a previous study has observed an increased popliteal arterial distensibility following 6 weeks of SIT (Rakobowchuk *et al.*, 2008). This suggests that sprint training can reduce arterial stiffness in the exercising limbs but maintains arterial stiffness centrally and in the untrained upper limbs in a healthy population.

Carotid arterial distensibility and IMT are linked to CVD progression (Simons *et al.*, 1999). In the present study, carotid arterial stiffness and IMT did not change following SIT or SCT in agreement with a previous study following SIT in healthy individuals (Rakobowchuk *et al.*, 2008). Healthy pre-training vasculature most likely explains the lack of training effect as carotid distensibility and IMT were similar to previously reported values in a healthy population (Tanaka *et al.*, 2002; Rakobowchuk *et al.*, 2008)

4.4.4 Training effect on CAC mobilisation and function

The present study is the first to evaluate the effects of sprint training on CAC mobilisation and function and demonstrated an increase in circulating $CD34^+$ cells following SIT and SCT but no change in its subpopulation $CD34^+CD45^{dim}$, suggesting that a different subpopulation of $CD34^+$ cells were mobilised. The enhanced $CD34^+$ cells did not correlate with FMD at pre-training or training induced changes in FMD indicating a healthy brachial artery endothelial function. Furthermore, cultured CACs adhesive and migratory ability did not alter following both training programmes. Conversely, increases in CAC function following exercise training have been documented in populations with or at risk of CVD (Sandri *et al.*, 2005; Steiner *et al.*, 2005; Sarto *et al.*, 2007). However, healthy individuals do not exhibit impaired CAC function (Vasa *et al.*, 2001), which likely explains why no increase in CAC function was observed in the present study. Furthermore, increases in CAC migratory ability occurred in those with lower pre-training levels in the present study, supporting the suggestion that exercise training increases CAC function only when individuals exhibit impaired CAC function at pre-training.

$CD34^+$ haematopoietic cells have been reported to migrate towards arterial injury (Walter *et al.*, 2002), adhere to implanted grafts (Shi *et al.*, 1998) and restore circulation to the ischaemic limb of mice (Hur *et al.*, 2004) providing evidence for their role in endothelial repair and angiogenesis. The present study is the first to show elevated numbers of CACs following exercise training in a healthy population and suggests an increased reparative potential if required. In contrast, no change in CACs was observed following 8 weeks of continuous endurance training in healthy older men (Thijssen *et al.*, 2006) and in our lab following 6 weeks of moderate intensity interval training in healthy young adults

(Rakobowchuk *et al.*, 2012). Although in the latter study, some participants exhibited a sustained mobilisation of CACs following heavy intensity interval training, which may have been a result of the higher exercise intensity. This evidence combined with the sustained mobilisation of CACs following both SIT and SCT in the present study suggests that the high intensity nature and not the duration or the interval vs. continuous nature of the exercise was the main contributor to these increases. This supports the theory that a greater metabolic stress during exercise leads to a sustained upregulation of CACs in healthy individuals as both training programmes in the present study involved maximal exertion sprints that likely achieved $\dot{V}O_{2max}$ (Whyte *et al.*, 2013). With increases in exercise intensity, greater levels of oxidative stress are produced (Goto *et al.*, 2003) which activates the endothelium, leading to secretion of VEGF and SDF-1α from endothelial cells that aid in the mobilisation and homing of CACs (Zampetaki *et al.*, 2008). Conversely, acute bouts of moderate-intensity exercise below the LT in healthy individuals have been shown to elevate CACs (Laufs *et al.*, 2005; Cubbon *et al.*, 2010) via a nitric oxide mediated pathway with levels returning to baseline after 24hrs. Taken together, these data suggests that for chronic increases in CACs following exercise training in healthy individuals, a higher level of oxidative and metabolic stress is required during exercise sessions.

4.4.5 Conclusions and future work

Sprint continuous training elicits similar increases in cardio-respiratory fitness and stem cell mobilisation as work-matched sprint interval training in a young healthy population, even though on average SCT session duration was only 3.5 min. However, brachial artery endothelial-dependent FMD may benefit more so following SIT due to the type or profile of shear stress experienced during the

exercise. The participation of such exercise throughout a women's lifespan may lead to a greater vascular health and quality of life at older age after the menopausal transition. Furthermore, increases in these variables in sedentary postmenopausal women or other populations at risk from CVD, might be of great benefit as these individuals exhibit impaired vascular function and repair. However, sprint training might not be appropriate for these populations as high motivation is required, and enjoyment will likely be low due to the feelings of nausea often associated with this type of maximal exercise. Therefore, future studies are warranted to adapt this type of training for women with or at risk of CVD such as postmenopausal women, by lowering the intensity. A recent study has started progress by adapting SIT for coronary artery disease patients and found increases in brachial artery FMD (Currie *et al.*, 2013). Training sessions were 2/week for 12 weeks and involved cycling at 90% of maximal HR for 60 s with a 60 s recovery period, repeated 10 times. However, other measures of vascular health such as arterial stiffness and CAC number and function following this type of training in CVD at risk populations have not been studied. Additionally, the intensity of the interval exercise cannot be confirmed using a percentage of HR_{max}, as explained in the next chapter. Thus, the following chapters investigate how interval exercise with short work and recovery periods effect vascular health and repair in postmenopausal women and obese women who display risk factors for CVD.

4.4.6 Study limitations

Comparisons between studies examining exercise induced CAC mobilisation is difficult due to the different antigens used to define cells and the various methods and gating strategies used for cell enumeration. This may explain why previous studies have shown no change in CAC number following exercise

training in healthy individuals whereas the present study reported an elevation. Inclusion of the endothelial cell specific marker VEGFR-2/KDR could not be included due to absence of the isotype control as discussed in the general methods, chapter 3, section 3.11.2. The addition of this antibody should be included in future studies (as seen in chapters 5 and 6) as it provides insight into the destination of the progenitor cells. Nevertheless, out of all its subpopulations, $CD34^+$ cells have been shown to be the best predictor of CVD, and therefore has an important role in maintaining vascular health (Fadini *et al.*, 2006b). We observed no alteration in CAC function following training however the paracrine function of these cells was not assessed. Addition of this measurement may have given insight into the impact the increase in $CD34^+$ cells had on the vasculature.

Finally, the duration of the training programme was relatively short (4 weeks); therefore it is unknown whether greater increases in the parameters measured may occur with further training. Future training studies (as seen in chapter 6) would benefit from a longer duration with measures assessed at different time points throughout the training programme.

Chapter 5 The effects of acute continuous and interval exercise on vascular function and repair in postmenopausal women

Aspects from this chapter were presented at the following conferences:

- European College of Sports Science, Annual meeting, Liverpool, UK, July 2011.
- European Society of Cardiology, EuroPRevent, Geneva, Switzerland, April 2011.
- American College of Sports Medicine's Conference on Integrative Physiology of Exercise, Miami Beach, Florida, September 2010.

And published in:

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5.1 Introduction

The risk of CVD in women significantly increases after the menopause due to the loss of oestrogen. The change in hormonal status additive to increasing age reduces endothelial function (Taddei *et al.*, 1996), increases arterial stiffness (Moreau *et al.*, 2003) and reduces the number and function of CACs (Fadini *et al.*, 2008). Current guidelines for women recommend lifestyle interventions such as exercise for prevention of CVD and myocardial infarction, rather than pharmacological approaches such as hormone replacement therapy due to the potential adverse side effects/risks associated with these drugs (Mosca *et al.*, 2011). Aerobic exercise training studies in postmenopausal women have demonstrated increases in endothelial function (Akazawa *et al.*, 2012; Swift *et al.*, 2012) and plasma nitrite/nitrate (Zaros *et al.*, 2009) and reductions in BMI

(Sugawara *et al.*, 2006), arterial stiffness (Moreau *et al.*, 2003), and oxidative stress (Attipoe *et al.*, 2008) which augment vascular health. Conversely, some studies have reported no change in endothelial function following exercise training in postmenopausal women (Casey *et al.*, 2007; Pierce *et al.*, 2008). These contrasting results might be explained by the different exercise methods and definitions of intensity used by different researchers.

Most if not all studies within the literature define exercise intensity using a percentage of $\dot{V}O_{2max}$, HR_{max} or HR reserve. This method does not accurately normalise the intensity of the exercise between participants as individuals exercising at the same percentage of $\dot{V}O_{2max}$ or HR_{max} can be above or below their individual LT (Meyer *et al.*, 1999). Similarly, individuals with the same $\dot{V}O_{2max}$ and cycling at the same percentage of $\dot{V}O_{2max}$ can be exercising in different exercise intensity domains [i.e. moderate, heavy, very heavy and severe Rossiter, (2011)]. Thus, the physiological stimulus for vascular adaptations may vary between participants which may lead to the discrepancy in results between studies or indeed between participants following the same intervention. Furthermore, the lack of control for intensity reduces the ability to identify the type and intensity of exercise that will yield the greatest benefits in health for specific populations, such as postmenopausal women. When different modes of exercise are compared such as interval vs. continuous, it is especially important to control for intensity in order to identify which type of exercise is better.

Recent evidence suggests that interval exercise is more or equally effective than the government guidelines of moderate-intensity continuous exercise for improving cardio-respiratory fitness, endothelial function and arterial stiffness (Wisloff *et al.*, 2007; Tjønna *et al.*, 2008; Ciolac *et al.*, 2010; Guimaraes *et al.*,

2010; Tordi *et al.*, 2010). Indeed, as seen in the previous chapter, interval type exercise appeared superior to a continuous mode for increasing brachial artery FMD. However, a comparison between interval and continuous exercise on these variables has not been investigated in postmenopausal women. Additionally, the effect of exercise *per se* on CAC number and function in postmenopausal women has not been studied. Furthermore, given that moderate-intensity is defined as exercise that does not cause a sustained accumulation in blood lactate in addition to $\dot{V}O_2$ falling at or below the LT (Rossiter, 2011), it is impossible to determine whether participants in both the interval and continuous groups in these previous studies, were exercising below their relative LT, and additionally if interval and continuous sessions were equally matched for intensity. Consequently, participants may have been exercising at a higher relative intensity in the interval sessions than the continuous sessions, which could explain the greater vascular improvements observed following interval exercise in some studies (Wisloff *et al.*, 2007; Schjerve *et al.*, 2008; Tjønna *et al.*, 2008; Ciolac *et al.*, 2010; Guimaraes *et al.*, 2010).

Therefore, the aims of this chapter were to 1) compare the acute effects of interval and continuous exercise, both within the moderate-intensity domain, on endothelial function, arterial stiffness and CAC number and function in post-menopausal women, and 2) compare these effects with a heavy-intensity interval session in a sub-set of participants to determine if a higher intensity stimulus has a greater impact on markers of vascular health. The advantages of investigating the acute effects of different types of exercise are two-fold. Firstly, it allows identification of exercise that has an immediate impact on markers of vascular health, which would be beneficial to individuals with poor vascular

health and secondly, it allows identification of the type and intensity of exercise that may yield the greatest improvements to vascular health if undertaken chronically. It was hypothesised that interval exercise would be superior to continuous exercise for improving markers of vascular health and repair due to the brief excursions to higher work-rates, and that due to the higher intensity, heavy-intensity interval exercise would exert greater effects.

5.2 Methods

5.2.1 Participants

Fifteen healthy postmenopausal women (age: 63 ± 4 yrs) volunteered for the study through poster and email advertisements placed around the local area. Postmenopausal status was defined as absence of menstrual cycle for at least 2 years and was confirmed through follicle stimulating hormone (FSH) greater than 30 $iU \cdot l^{-1}$ (refer to section 5.2.3). Exclusion criteria other than specified in the general methods, chapter 3, section 3.1, included exercising greater than twice per week and was confirmed orally prior to participation. A resting 12-lead ECG was used at pre-testing and throughout the maximal exercise test to confirm absence of ECG abnormalities.

5.2.2 Experimental protocol

For the assessment and calculation of work-rate for the exercise bouts, participants attended the University of Leeds exercise physiology laboratory on two occasions, each separated by one week. On visit one vascular measures and a fasted blood sample (~60 ml) for the assessment of blood markers and CAC number and function were completed. On the second visit a cardio-respiratory fitness test was completed for the assessment of peak aerobic capacity ($\dot{V}O_{2peak}$) and the LT to enable calculation of the work-rates for the subsequent exercise bouts. Control factors for these two sessions are specified

in the general methods, chapter 3, section 3.2. Following these visits, participants attended the laboratory on two further occasions, each separated by ≥1 week for completion of a 30 min moderate-intensity continuous and a 30 min moderate-intensity interval exercise bout on a cycle ergometer. Once these sessions had been completed the study was extended to compare the acute effects of a heavy-intensity interval exercise bout on a cycle ergometer. For this second phase, a sub-set of participants (n = 9) attended the laboratory on one further occasion for completion of a 30 min heavy-intensity interval exercise bout. Participants refrained from consuming food and caffeine in the 2 hours prior to the exercise session visits. To assess the acute effects of each of the exercise bouts on markers of vascular health, the vascular measures were assessed pre and 15 min post-exercise. To assess the acute exercise effects on CAC number and function, a ~50 ml fasted blood sample was acquired 30 min post-exercise and results compared with that of visit one. A schematic of the protocol can be viewed in Figure 5.1.

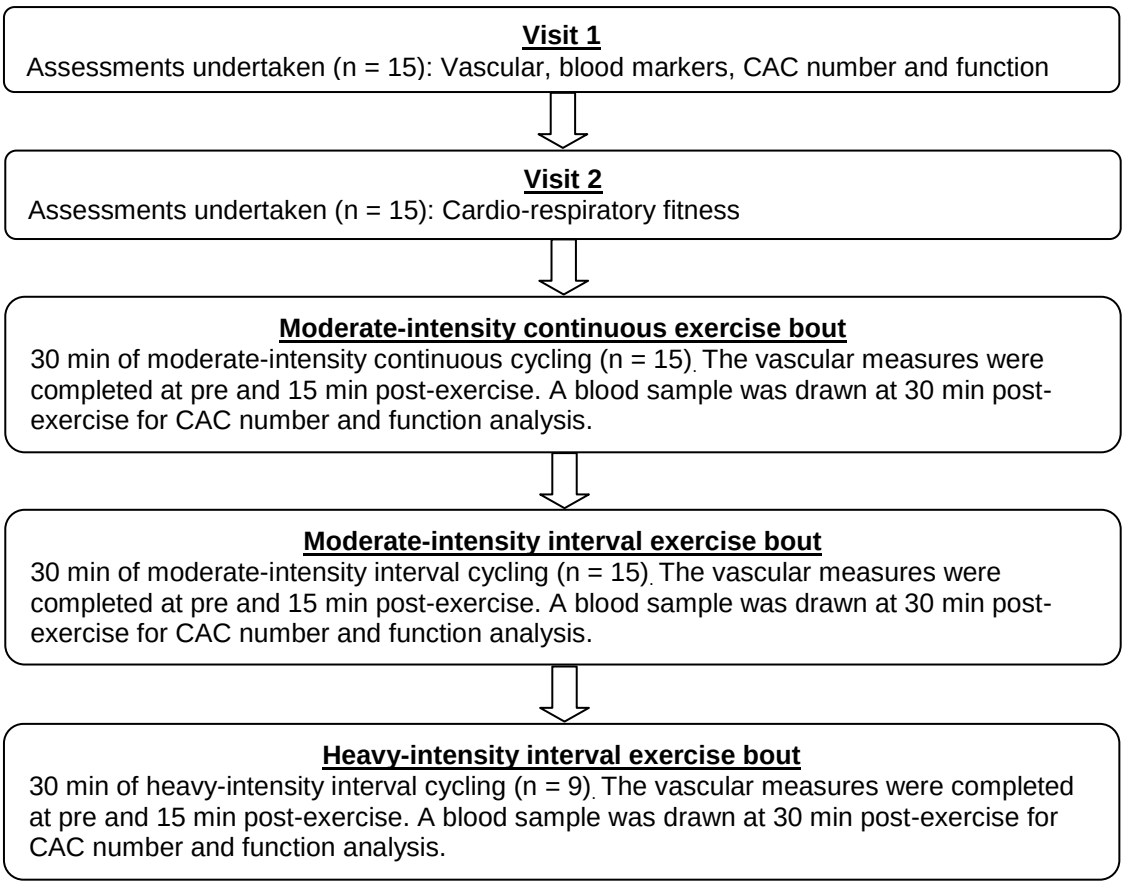

Figure 5.1. A flow-chart of the experimental protocol.

5.2.3 Variables assessed pre and post-exercise session

The protocols for the following variables are described in detail in the general methods chapter (chapter 3).

5.2.3.1 Cardio-respiratory fitness

A seated ramp-incremental exercise test with a ramp-rate of 10 W/min was performed for the assessment of $\dot{V}O_{2peak}$ and the LT (section 3.3). and for calculation of the work-rates achieved at these points for the subsequent exercise sessions (i.e. WR_{peak} and WR_{LT}).

5.2.3.2 Vascular measures

The following vascular measures were completed on visit one and at pre and post-exercise: BMI (section 3.4.1), brachial artery endothelial function for the measurement of FMD, peak reactive hyperaemia, peak shear rate, AUC_{peak},

AUC_{60} and AUC_{90} and their corresponding VTIs (section 3.5), brachial artery blood pressure (section 3.7), carotid arterial stiffness for assessment of cross-sectional compliance (CSC), distensibility, and β-stiffness index (section 3.8). During recording of brachial artery blood velocity the insonation angle for each participant between pre and post-testing and between different exercise visits was within 2°.

5.2.3.3 Blood markers and CAC number and function

A fasted blood sample was drawn on visit one and post-exercise following the protocol in section 3.9. A 50 ml blood sample was collected and divided into EDTA vacutainers for analysis of CAC number and function. On visit one only an extra 10 ml of blood was collected in either serum vacutainers containing a clot accelerator or plasma EDTA vacutainers and sent to the Leeds General Infirmary Pathology services to test for serum FSH levels, cholesterol profile, insulin, and plasma glucose and haemoglobin A1c (HbA1c). CACs were enumerated from 21 ml of blood via flow cytometry using a commercially available kit (EPC enrichment and enumeration kit, Miltenyi Biotec; section 3.11.2.1). CACs were defined as $CD34^+$, double positive ($CD34^+KDR^+$) or triple positive ($CD34^+KDR^+CD133^+$). To assess the *in vitro* function of CACs, the ability to migrate to a chemoattractant, adhere to fibronectin and to form CFUs was determined. The detailed protocol for cell culture and the functional assays are described in the general methods chapter sections 3.12.1-3.12.4. Briefly, 25 ml of blood was mixed with 25 ml of PBS and the mononuclear cells separated by ficoll density-gradient centrifugation. For quantification of cell phenotype adherent CACs were double stained with DiI-AcLDL and lectin on day 7. For the migration and adhesion assays, cells were cultured for 7 days with daily medium changes. Assays were performed in triplicate and an average

calculated if the required number of adherent cells on day 7 were harvested. If insufficient cells were harvested the assay was performed in duplicate or individually. For CFU assessment, CACs were cultured for 48 hrs initially. The non-adherent cells were then replated and cultured for a further 72 hrs, whereupon the number of CFUs were counted per well.

5.2.4 Exercise session protocols

All participants completed a 30 min moderate-intensity continuous (CON) and a 30 min moderate-intensity interval (MOD INT) exercise bout on a cycle ergometer (Lode BV, Excalibur Sport V2.0, the Netherlands) on two separate days. A sub-set of 9 participants completed a third session involving a 30 min heavy-intensity interval (HEAVY INT) exercise bout to compare the effects following a higher intensity exercise session.

The CON exercise bout involved cycling at 80% of WR_{LT} and was therefore in the moderate-intensity domain as $\dot{V}O_2$ was below the LT (Rossiter, 2011). The MOD INT and HEAVY INT exercise bouts were based on a study by Turner *et al*., (2006), which investigated the physiological responses to different work:recovery duty cycles, and identified the interval exercise sessions that represent exercise in the moderate and heavy-intensity domains. Therefore, in the present study, this enabled CON exercise to be matched with INT for the moderate-intensity domain, and for the subsequent comparison with heavy-intensity INT exercise. In this previous study, four duty cycles were studied in four separate 30 min exercise sessions. All sessions were conducted with a 1:2 work:recovery ratio at 120% WR_{peak} with the recovery periods undertaken at 20 W. Given that all sessions were of an equal duration and utilised the same work-rates; the work completed in all sessions was identical. The authors recorded HR, $\dot{V}O_2$ and blood lactate throughout each session with

work:recovery ratios of 10:20, 30:60, 60:120, 90:180 s. The results indicated that the 10:20 s duty cycle presented characteristics of the moderate-intensity domain, as blood lactate accumulation was not sustained and average $\dot{V}O_2$ was not significantly greater than the LT throughout the 30 min session (Figure 5.2a,e). The 30:60 s duty cycle presented characteristics of heavy-intensity continuous exercise as the peaks of the $\dot{V}O_2$ profile increased from rest and stabilised at a level above the LT at ~10-15 min. Blood lactate increased above resting levels and plateaued at an elevated level from ~10 min (Figure 5.2b,e). The 60:120 s and 90:180 s duty cycles were characteristic of the very-heavy and severe exercise intensity domains, as blood lactate and the peaks of the $\dot{V}O_2$ profile continued to increase throughout the sessions, and not all participants could complete the entire 30 min session (Figure 5.2c-e). For the purposes of the present study, the acute INT exercise bouts were required to be both above and below the LT. As such, the chosen duty cycles for the MOD INT and HEAVY INT exercise bouts were 10:20 s and 30:60 s duty cycles, respectively. However, in the present study a population of sedentary postmenopausal women were studied as opposed to the study by Turner *et al.,* (2006), which was conducted in healthy young males. Therefore, the work-rate was reduced to 90% WR_{peak} assuming that the physiological responses to the 10:20 and 30:60 s duty cycle exercise sessions would be characteristic of the moderate and heavy-intensity domains, respectively. Brachial artery blood pressure and heart rate was recorded in 5 min intervals throughout each session using a manual sphygmomanometer and a polar heart rate monitor (Kempele, Finland), respectively.

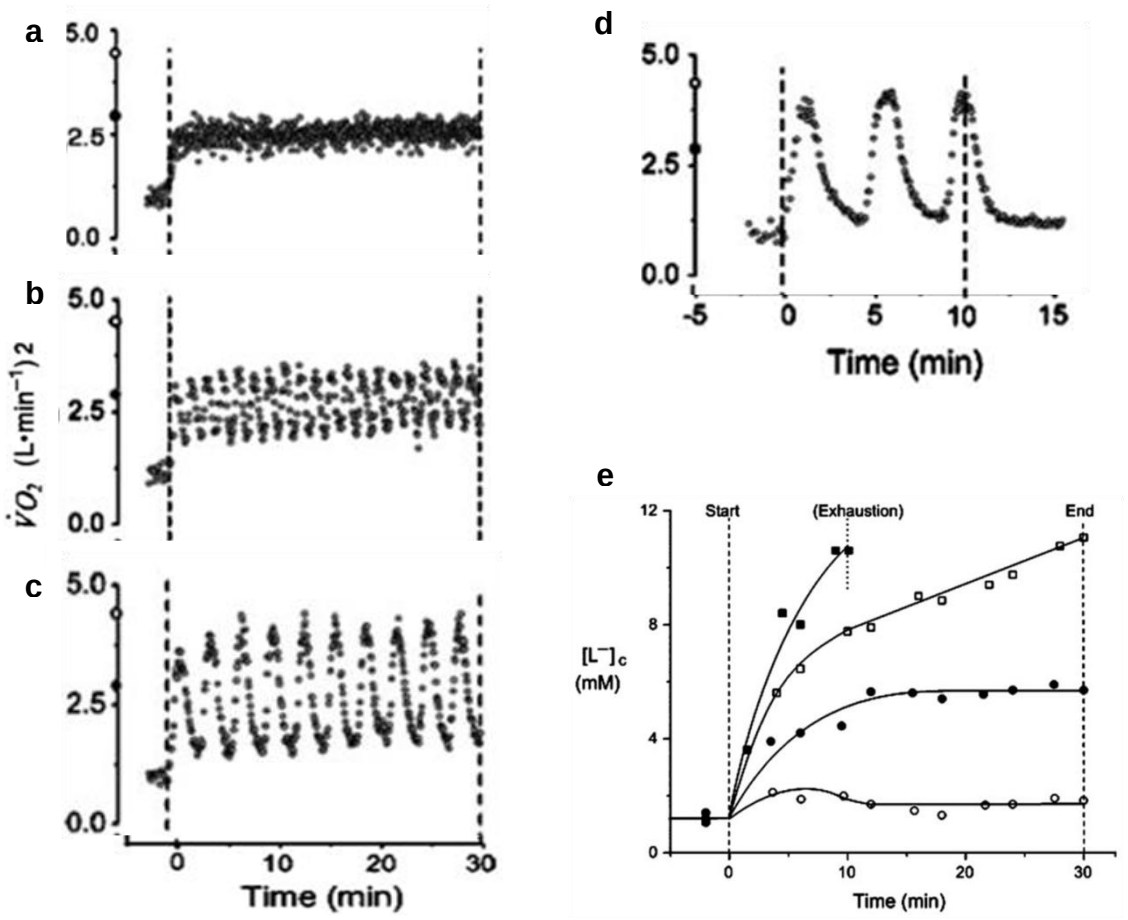

Figure 5.2. Representation of the $\dot{V}O_2$ and blood lactate responses to different interval exercise duty cycles. During the 10:20 s session the $\dot{V}O_2$ rises to a level around the LT (a; 2.05 L·min^{-1}) and there is no blood lactate accumulation (e; open circles). During the 30:60 s session the $\dot{V}O_2$ peaks increase above the LT and plateau (b) and the blood lactate significantly rises above resting levels but remains constant (e; closed circles). During the 60:120 s session the $\dot{V}O_2$ peaks reach close to $\dot{V}O_{2max}$ and increase through the session (c) and blood lactate increases continuously throughout the session (e; open squares). Participants could not complete the 30 min duration of the 90:180 s session due to $\dot{V}O_{2max}$ attained (d) and the blood lactate levels steeply increasing from exercise onset (e; closed squares). Modified from Turner *et al*., (2006).

5.2.5 Statistical analysis

There were no differences in all variables at pre-exercise between the visits. A non-parametric Friedman's ANOVA was conducted on CAC number only as data was not normally distributed and could not be corrected using transformation. The data were examined by 1) a two-way within-subject

repeated measures ANOVA for the moderate-intensity exercise bouts with time (pre vs. post-exercise) and exercise type (CON, MOD INT) treated as the independent variables, and 2) in the sub-set of 9 participants an additional ANOVA was performed to analyse the acute effects of a heavy-intensity INT exercise bout. Thus, the independent variable exercise type included CON, MOD INT and HEAVY INT. In one participant at the CON exercise and two participants at MOD INT exercise session, post-exercise FMD was compared with visit one due to poor image quality at pre-exercise. Paired t-tests were conducted for post-hoc analysis to identify which exercise bout showed a significant change. Paired t-tests were performed on exercise session average work-rates and work done to compare differences between the CON and INT exercise bouts. Due to the experimental design, the MOD and HEAVY INT exercise bouts were matched for average work-rate and work done, therefore comparisons between these two sessions were not required. Given the potential different responses in brachial artery FMD following interval and continuous type exercise observed in the previous chapter, brachial artery FMD was selected as the primary outcome. Using the previously reported acute increase of 4.6% in brachial artery FMD, following 45 min of treadmill exercise in postmenopausal women (Harvey *et al.*, 2005), and a standard deviation of 3% (reported from healthy women in chapter 4), a minimum of 9 participants in total were required to obtain 80% power ($\alpha = 0.05$) in a two-treatment crossover study.

Several experimental issues were experienced during the study which resulted in participants excluded from analysis. These issues are listed below;

- Poor image quality of the ultrasound images
- Flow cytometry error/failure

- Low cell numbers separated at the start of cell culture
- Insufficient cells harvested on day 7 of cell culture

Therefore, the number of participants included in each analysis within this chapter is specified for each measure.

5.3 Results

5.3.1 Participant and exercise session characteristics

Participant characteristics are displayed in Table 5.1. Postmenopausal status was confirmed as FSH levels were >30 $iU \cdot l^{-1}$. Total cholesterol and LDL were higher than the desirable healthy range (>5.2 $mmol \cdot l^{-1}$ and >3.4 $mmol \cdot l^{-1}$, respectively).

Table 5.1. Participant characteristics of postmenopausal women from visit one.

	n	Visit one (Mean ± SD)
Age (yrs)	15	63 ± 4
BMI ($kg \cdot m^{-2}$)	15	25.0 ± 3.1
Brachial artery SBP (mmHg)	15	137 ± 15
Brachial artery DBP (mmHg)	15	84 ± 5
Brachial artery MAP (mmHg)	15	102 ± 8
Plasma glucose ($mmol \cdot l^{-1}$)	13	4.8 ± 0.5
HbA1c ($mmol \cdot mol\ HB^{-1}$)	14	39 ± 2
Total cholesterol ($mmol \cdot l^{-1}$)	14	5.9 ± 1.0
HDL ($mmol \cdot l^{-1}$)	14	1.9 ± 0.5
LDL ($mmol \cdot l^{-1}$)	14	3.5 ± 0.8
Cholesterol:HDL ratio	14	3.1 ± 0.7
Triglycerides ($mmol \cdot l^{-1}$)	14	1.0 ± 0.4
FSH ($iU \cdot l^{-1}$)	13	69.9 ± 31.0
Insulin ($mU \cdot l^{-1}$)	14	6.5 ± 3.1

Reduced n (number of participants) for specific variables due to blood tests showing erroneous results. BMI = body mass index, SBP = systolic blood pressure, DBP = diastolic blood pressure, MAP = mean arterial pressure, HbA1c = haemoglobin A1c, HDL = high-density lipoprotein, LDL = low-density lipoprotein and FSH = follicle stimulating hormone.

All 15 participants completed the CON and MOD INT exercise sessions with 9 participants completing a further HEAVY INT exercise session. Participants absolute and relative $\dot{V}O_{2peak}$ were 1.40 ± 0.29 $L \cdot min^{-1}$ and 21.6 ± 5.4 $ml \cdot kg \cdot min^{-1}$, respectively. The within-exercise session characteristics are displayed in Table 5.2. Average work-rate for the interval sessions was calculated from 70%Δ work-rate and the recovery periods of 20 W. By design the average work-rate and work done during the MOD INT and HEAVY INT exercise sessions were equal. The average work-rate and work done was significantly higher in the CON exercise sessions when compared with the INT sessions (p = 0.001, Table 5.2).

Table 5.2. Exercise session characteristics (mean ± SD) for the continuous (CON) and interval (INT) exercise sessions.

	CON	MOD INT	HEAVY INT
Average WR (W)	35 ± 6	30 ± 1.5 *	30 ± 1.5 *
Work done (kJ)	63.9 ± 10.1	54.0 ± 2.8 *	54.0 ± 2.8 *
Average session HR (bpm)	107 ± 13	103 ± 12	103 ± 9
Average session SBP (mmHg)	157 ± 17	150 ± 16	149 ± 20
Average session DBP (mmHg)	83 ± 6	82 ± 5	80 ± 6

***** indicates a significant difference to the CON exercise session (p < 0.05). $\dot{V}O_{2peak}$ = peak oxygen uptake, RI = ramp incremental, WR = work-rate, HR = heart rate, SBP = systolic blood pressure, DBP = diastolic blood pressure.

5.3.2 Brachial artery endothelial function in postmenopausal women following a 30 min bout of continuous and interval exercise

FMD was not normalised to shear rate as a significant correlation was not observed between absolute FMD and peak shear rate, AUC_{peak}, AUC_{60} and AUC_{90} (p > 0.05). Brachial artery endothelial function did not change following an acute bout of CON and MOD INT exercise in 14 postmenopausal women (p > 0.05, Table 5.3). There were no significant changes in brachial artery resting diameter (p = 0.53), time from cuff deflation to peak diameter (p = 0.86),

absolute FMD (p = 0.49), relative FMD (p = 0.90), VTI_{peak} (p = 0.44), VTI_{60} (p = 0.32), VTI_{90} (p = 0.40), peak reactive hyperaemia (p = 0.52), peak shear rate (p = 0.29), shear rate AUC_{peak} (p = 0.44), shear rate AUC_{60} (p = 0.27) and shear rate AUC_{90} (p = 0.34). Additionally, there were no time by exercise type interactions in these variables (p > 0.05).

Table 5.3. Brachial artery endothelial function (mean ± SD) pre and post an acute 30 min bout of moderate-intensity continuous (CON) and interval (MOD INT) exercise (n = 14) and heavy-intensity interval (HEAVY INT) exercise (n = 8). Data reported from two separate ANOVAs.

	CON		MOD INT		Heavy INT	
	Pre	Post	Pre	Post	Pre	Post
Resting diameter (mm)	3.5 ± 0.4	3.4 ± 0.4	3.5 ± 0.4	3.4 ± 0.4	3.3 ± 0.5	3.4 ± 0.4
Time from cuff release to peak diameter (s)	46 ± 17	50 ± 23	54 ± 17	51 ± 18	69 ± 21	77 ± 29
Insonation angle (°)	68 ± 1	68 ± 1	68 ± 1	68 ± 1	68 ± 1	68 ± 1
Absolute FMD (mm)	0.22 ± 0.08	0.19 ± 0.12	0.19 ± 0.08	0.18 ± 0.09	0.16 ± 0.04	0.14 ± 0.06
VTI_{peak} (cm)	1471 ± 614	1720 ± 700	1681 ± 597	1719 ± 709	1937 ± 942	2250 ± 817
VTI_{60} (cm)	1660 ± 501	1898 ± 424	1806 ± 435	1861 ± 410	1851 ± 884	1945 ± 452
VTI_{90} (cm)	2135 ± 665	2385 ± 618	2281 ± 564	2364 ± 579	2234 ± 1112	2425 ± 606
Peak reactive hyperaemia ($cm \cdot s^{-1}$)	72.8 ± 27.7	78.2 ± 28.1	87.6 ± 32.8	87.4 ± 24.8	96.5 ± 41.4	102.4 ± 24.6
Peak shear rate (s^{-1})	1681 ± 658	1850 ± 734	2072 ± 892	2096 ± 723	2349 ± 1020	2463 ± 621
AUC_{peak} (a.u.)	33803 ± 15453	39766 ± 14131	39477 ± 15399	40027 ± 14399	46578 ± 21982	53954 ± 19873
AUC_{60} (a.u.)	38342 ± 13224	44365 ± 9434	42292 ± 11723	44053 ± 9443	44430 ± 20151	46649 ± 11587
AUC_{90} (a.u.)	49228 ± 17293	55511 ± 12836	53211 ± 14127	55796 ± 12521	53401 ± 24891	57871 ± 14281

FMD = flow-mediated dilation, VTI = velocity-time integral, AUC = shear rate area under the curve.

In the HEAVY INT analysis in the subset of participants, 8 participants were included in the analysis. There were no significant changes following any exercise bout and no time by exercise type interactions (Table 5.3) in brachial

artery resting diameter (p = 0.76), time from cuff deflation to peak diameter (p = 0.68), absolute FMD (p = 0.85), relative FMD (p = 0.80, Figure 5.3), VTI_{peak} (p = 0.37), VTI_{60} (p = 0.33), VTI_{90} (p = 0.26), peak reactive hyperaemia (p = 0.42), peak shear rate (p = 0.33), shear rate AUC_{peak} (p = 0.33), shear rate AUC_{60} (p = 0.25) and shear rate AUC_{90} (p = 0.19).

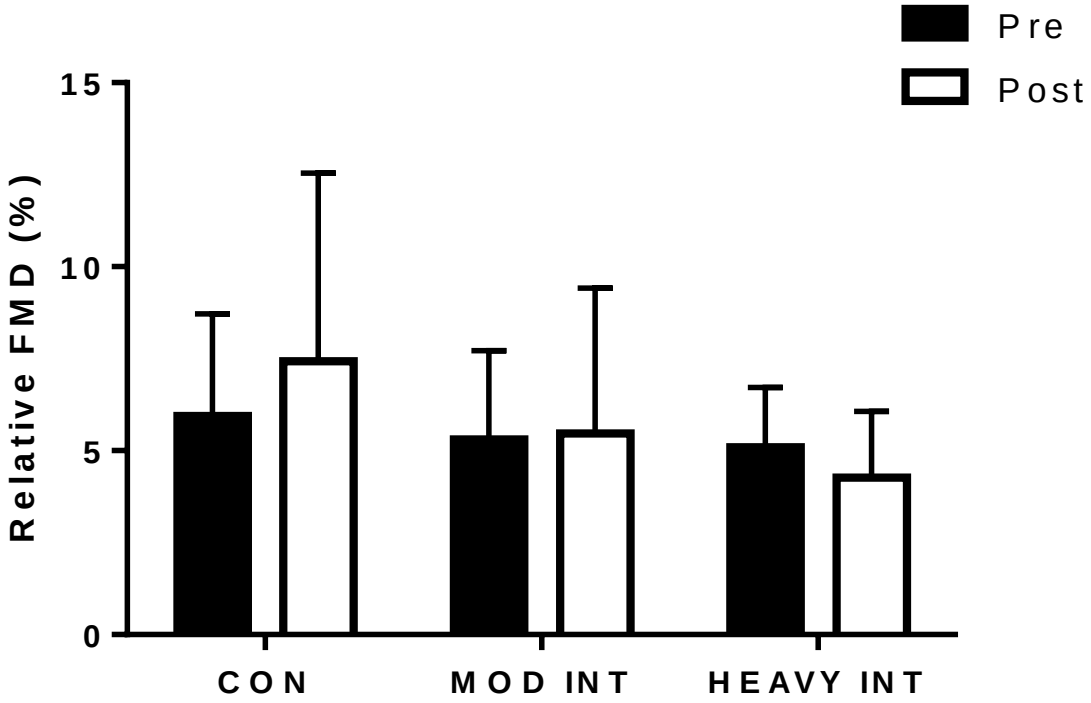

Figure 5.3. Brachial artery flow-mediated dilation (FMD, mean ± SD) pre and post a 30 min bout of moderate-intensity continuous (CON), interval (MOD INT) and heavy-intensity interval (HEAVY INT) exercise (n = 8, p > 0.05). No acute exercise effects were observed (p > 0.05).

5.3.3 Carotid arterial stiffness in postmenopausal women following a 30 min bout of continuous and interval exercise

Blood pressure and carotid arterial stiffness did not change following an acute bout of CON and MOD INT exercise in 15 postmenopausal women (p > 0.05). There were no significant changes after either exercise bout in brachial artery SBP (p = 0.35), DBP (p = 0.17), MAP (p = 0.14), and PP (p = 0.86) and carotid artery SBP (p = 0.13), PP (p = 0.32), delta CSA (p = 0.82), CSC (p = 0.65), distensibility (p = 0.45) and SI (p = 0.43). Additionally, there were no significant time by exercise type interactions (p > 0.05).

Table 5.4. Brachial artery blood pressure and carotid arterial stiffness (mean ± SD) pre and post an acute 30 min bout of moderate-intensity continuous (CON) and interval (MOD INT) exercise and heavy-intensity interval (HEAVY INT) exercise (n = 9). No acute exercise effects were observed ($p > 0.05$).

	CON		MOD INT		HEAVY INT	
	Pre	Post	Pre	Post	Pre	Post
Brachial artery DBP (mmHg)	85 ± 6	81 ± 6	81 ± 7	81 ± 9	83 ± 5	83 ± 6
Brachial artery MAP (mmHg)	101 ± 7	99 ± 7	99 ± 11	98 ± 11	100 ± 6	102 ± 8
Carotid ΔCSA (mm^2)	4.4 ± 1.6	4.6 ± 1.1	4.2 ± 1.3	4.1 ± 1.2	4.5 ± 1.9	4.2 ± 1.4
Carotid artery CSC (mm^2/mmHg)	0.12 ± 0.04	0.12 ± 0.02	0.10 ± 0.04	0.11 ± 0.03	0.11 ± 0.04	0.10 ± 0.03
Carotid artery distensibility (mm/mmHg)	0.003 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.003 ± 0.001
Carotid artery SI (a.u.)	6.8 ± 1.9	6.4 ± 1.3	8.1 ± 2.8	7.3 ± 2.0	7.3 ± 2.4	8.2 ± 2.2

DBP = diastolic blood pressure, MAP = mean arterial pressure, ΔCSA = delta cross-sectional area, CSC = cross-sectional compliance, SI = β-stiffness index.

Inclusion of the HEAVY INT exercise session into the ANOVA revealed that there were also no changes or time by exercise type interactions ($p < 0.05$, Table 5.4) in brachial artery DBP ($p = 0.60$), MAP ($p = 0.62$), and carotid artery delta CSA ($p = 0.85$), CSC ($p = 0.71$), distensibility ($p = 0.75$) and SI ($p = 0.88$). However, there was a significant time by exercise type interaction in brachial artery SBP ($p = 0.01$), PP ($p = 0.01$) and carotid artery SBP ($p = 0.02$) and PP ($p = 0.01$). As shown in Figure 5.4, brachial artery SBP ($p = 0.04$), PP ($p = 0.06$) and carotid artery SBP ($p = 0.08$) were elevated 15 min after cessation of HEAVY INT exercise by 6 mmHg, 5 mmHg and 4 mmHg, respectively. In contrast following MOD INT exercise, brachial artery PP ($p = 0.06$) and carotid artery PP ($p = 0.04$) reduced by 5 mmHg and 7 mmHg, respectively. There were no changes following CON exercise ($p > 0.05$). Participants with a higher brachial artery PP and carotid artery SBP and PP before exercise had a greater reduction in brachial artery PP ($r = -0.72$, $p = 0.03$, $n = 9$) and carotid artery

SBP (r = -0.67, p = 0.048, n = 9) and PP (r = -0.84, p = 0.04, n = 9) following the MOD INT exercise session only.

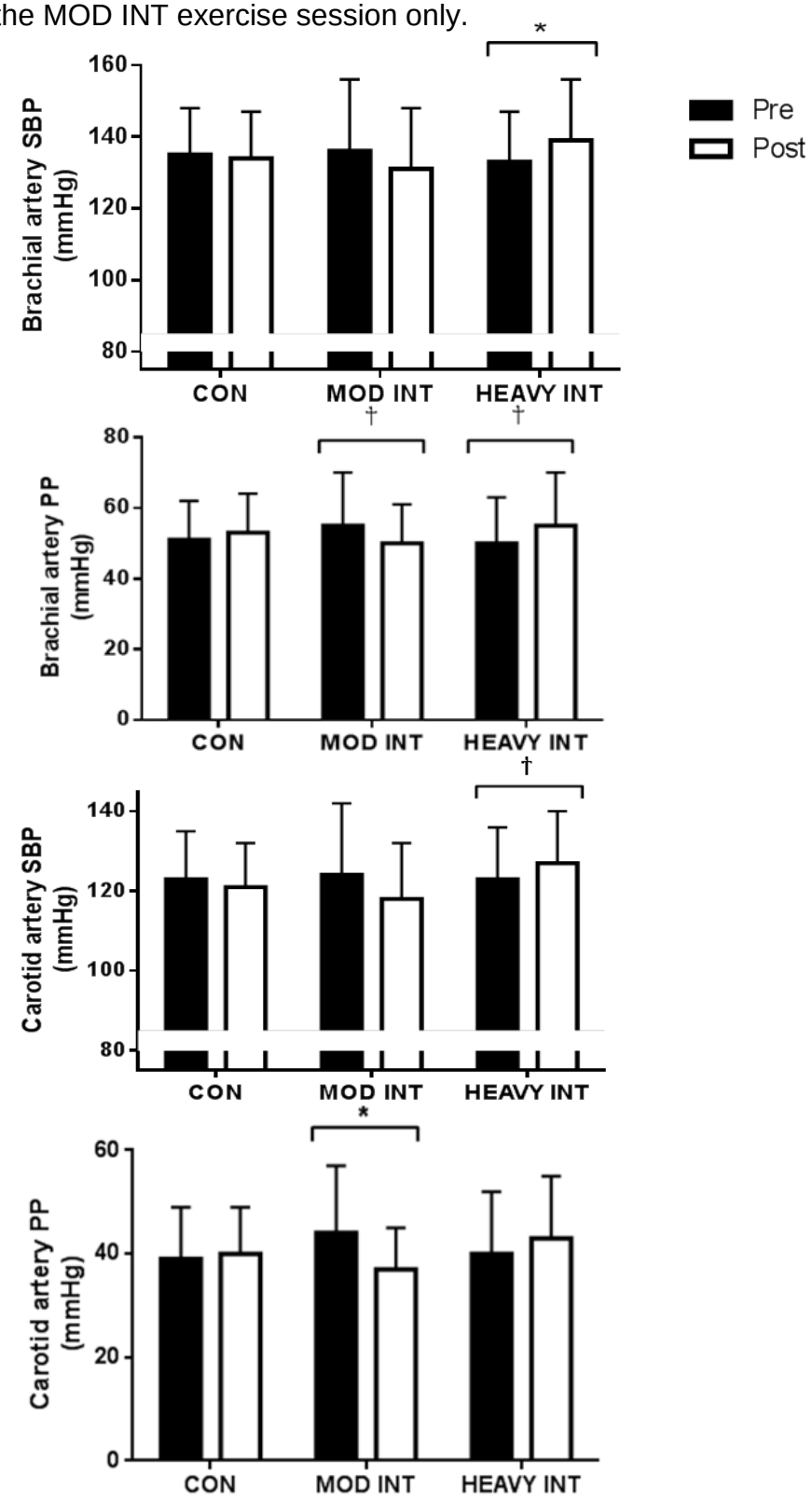

Figure 5.4. Brachial and carotid artery systolic (SBP) and pulse pressure (PP, mean ± SD) pre and post a 30 min bout of moderate-intensity continuous (CON), interval (MOD INT) and heavy-intensity interval (HEAVY INT) exercise (n = 9). * indicates a significant difference between pre and post-exercise (p < 0.05). † indicates a close to significant trend between pre and post-exercise (p < 0.08). There was a significant time by exercise type interaction (p < 0.05) in all measures.

5.3.4 CAC number in postmenopausal women following a 30 min bout of continuous and interval exercise

The number of $CD34^+$, $CD34^+KDR^+$ and $CD34^+KDR^+CD133^+$ circulating angiogenic cells were enumerated following each exercise session. There was no significant change following the CON and MOD INT exercise bout (n = 9) in $CD34^+$ cells (p = 0.28), $CD34^+KDR^+$ cells (p = 0.57) and $CD34^+KDR^+CD133^+$ cells (p = 0.74). Additionally, there was no significant change in the subset of 6 participants following the HEAVY INT bout in all CAC populations (p > 0.05, Table 5.5).

Table 5.5. Circulating angiogenic cell number (mean ± SD) at visit one and post an acute 30 min bout of moderate-intensity continuous (CON) and interval (MOD INT) exercise and heavy-intensity interval (HEAVY INT) exercise (n = 6).

	Visit one	CON	MOD INT	HEAVY INT
$CD34^+$ cells /10 ml blood	279672 ± 133899	219226 ± 122865	263816 ± 110710	231205 ± 91468
$CD34^+KDR^+$ cells /10 ml blood	251 ± 176	108 ± 119	244 ± 188	144 ± 201
$CD34^+KDR^+CD133^+$ cells /10 ml blood	68 ± 102	11 ± 13	40 ± 85	14 ± 26

5.3.5 CAC function in postmenopausal women following a 30 min bout of continuous and interval exercise

The ability of cultured cells to uptake DiI-AcLDL and lectin was quantified on day 7 of cell culture. There was no significant change following the CON and MOD INT exercise bout (p = 0.42, n = 10, Table 5.6) and in the subset of 5 participants following the HEAVY INT bout (p = 0.58) and no time by exercise type interactions (p > 0.05). Due to the low n value (n = 5) in the ANOVA and a greater number of DiI/lectin CACs following HEAVY INT, paired t-tests were performed on the exercise bouts, which revealed a significant increase following

HEAVY INT exercise (n = 7, *p* = 0.02, Figure 5.5) whereas there were no changes after CON and MOD INT exercise (n = 11, *p* > 0.05),

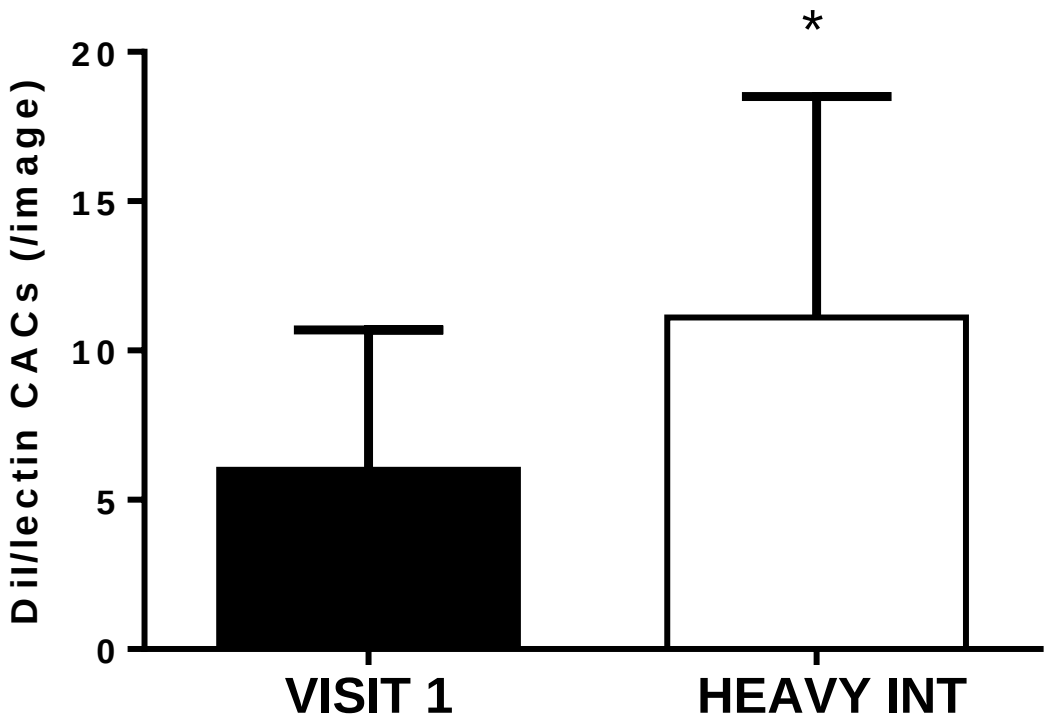

Figure 5.5. The number (mean ± SD) of DiI-AcLDL and lectin stained CACs after 7 days of culture at visit one and following a 30 min bout of heavy-intensity interval exercise (HEAVY INT).* indicates a significant difference between visit one (*p* < 0.05).

5.3.5.1 The migratory ability of CACs in postmenopausal women following a 30 min bout of continuous and interval exercise

To assess the functional ability of cultured CACs to migrate to a chemoattractant after 24 hrs, the number of cells migrated towards a control vehicle was subtracted from the number of cells migrated towards VEGF. In several participants the value was negative (i.e. the number of migrated cells was higher towards the control vehicle). In these cases a zero value was reported. The migratory ability of CACs did not change following the CON and MOD INT exercise bout (*p* = 0.62, n = 13, Table 5.6) or in the subset of 6 participants following the HEAVY INT bout (*p* = 0.38, Figure 5.6) and no time by exercise type interactions were observed (*p* > 0.05). However, there was a

large effect size for a decrease in CAC migration following HEAVY INT exercise (d = 0.9).

Table 5.6. Circulating angiogenic cell (CAC) function (mean ± SD) at visit one and post an acute 30 min bout of moderate-intensity continuous (MOD CON) and interval (INT) exercise. Data reported from the moderate-intensity ANOVA.

	Visit one	CON	MOD INT
DiI-AcLDL & lectin CACs/ microscopic image (n = 10)	6 ± 5	5 ± 3	7 ± 5
CAC migration/10 microscopic images (n = 13)	4 ± 4	4 ± 3	5 ± 6
CAC adhesion/ microscopic image (n = 12)	8 ± 6	7 ± 5	8 ± 5
CFUs /well (n = 14) **	12 ± 10	13 ± 14	32 ± 30

** indicates a significant time effect and time by exercise type interaction (p < 0.05).

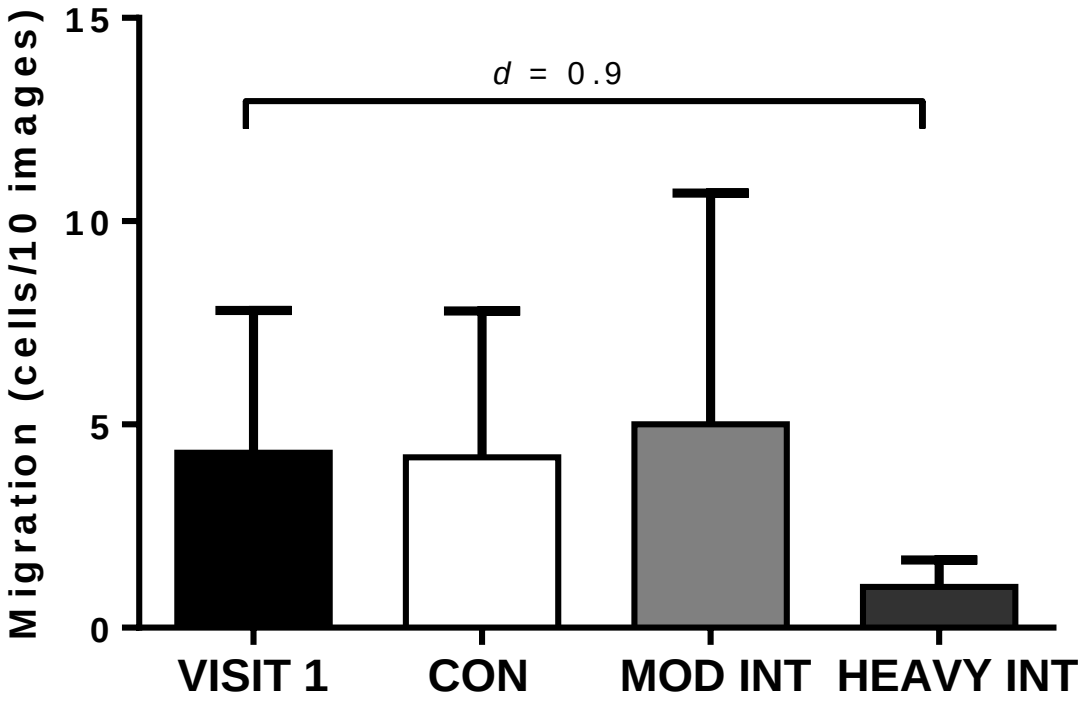

Figure 5.6. The number (mean ± SD) of migrated CACs to VEGF at visit one and following a 30 min moderate-intensity continuous (CON), moderate-intensity interval (MOD INT) and heavy-intensity interval (HEAVY INT) exercise bout (n = 6). The ability of CACs to migrate did not change following either exercise bout (p = 0.38) and there was no time by exercise type interaction (p = 0.15). Cohen's d = effect size.

5.3.5.2 The adhesive ability of CACs in postmenopausal women following a 30 min bout of continuous and interval exercise

To assess the adhesive ability of CACs following an acute exercise bout the number of cultured CACs that adhered to fibronectin over 24 hrs was counted. The adhesive ability of CACs did not change following the CON and MOD INT exercise bout (p = 0.98, time by exercise p = 0.70, n = 12, Table 5.6) or in the subset of 7 participants following the HEAVY INT bout (p = 0.60). There was a close to significant time by exercise type interaction between the three exercise sessions (n = 7, p = 0.06, Figure 5.7). However, paired t-tests revealed no significant difference between visit one and post-exercise in all sessions ($p > 0.05$), and the effect sizes for an increase in CAC adhesion following HEAVY INT and a decrease following MOD INT were modest (Figure 5.7).

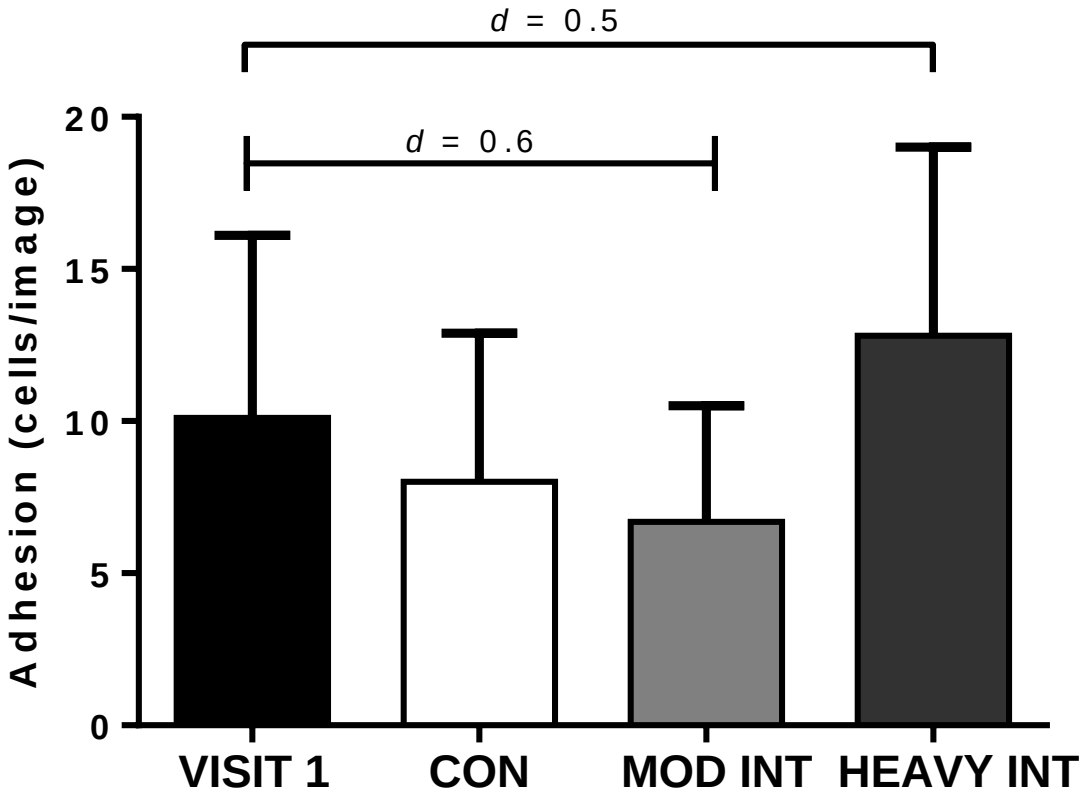

Figure 5.7. The number (mean ± SD) of adhered CACs to fibronectin at visit one and following a 30 min moderate-intensity continuous (CON), moderate-intensity interval (MOD INT) and heavy-intensity interval (HEAVY INT) exercise bout (n = 7). There was no time effect (p = 0.60) but a close to significant time by exercise type interaction (p = 0.06).

5.3.5.3 The number of CFUs in postmenopausal women following a 30 min bout of continuous and interval exercise

Comparisons between the CON and MOD INT exercise session (n = 14) revealed a significant time effect (p = 0.02) with a significant increase following the MOD INT exercise session but no change following the CON exercise session (time x exercise type p = 0.02, Table 5.6). There was a significant time effect (p = 0.007) in the sub-set of 9 participants following the CON, MOD INT and HEAVY INT exercise sessions and a significant time by exercise type interaction (p = 0.01, Figure 5.8). Paired t-tests revealed a significant increase from visit one to post MOD INT by 139% (n = 9, p = 0.045) and post HEAVY INT by 238% (n = 9, p = 0.01) but no change following CON exercise (n = 9, p = 0.60, Figure 5.8). There was no significant difference between the change from visit one to MOD INT and from visit one to HEAVY INT (n = 9, p = 0.32). This suggests that interval exercise increases CFUs regardless of intensity but moderate-intensity continuous exercise has no effect.

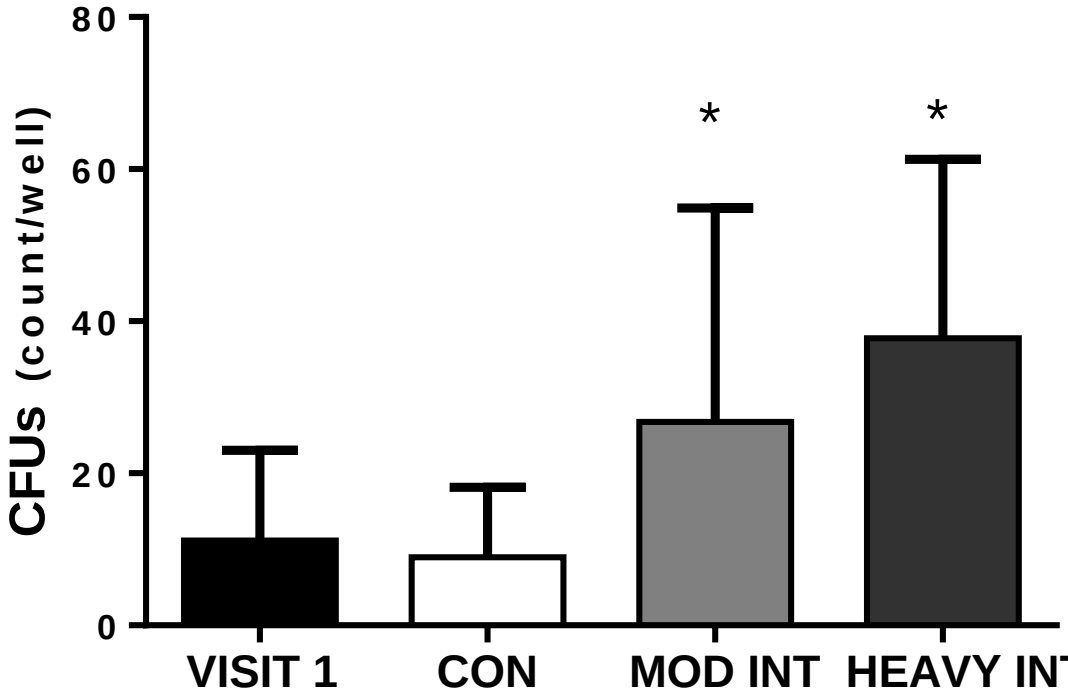

Figure 5.8. The number (mean ± SD) of colony-forming units (CFUs) at visit one and following a 30 min moderate-intensity continuous (CON), moderate-intensity interval (MOD INT) and heavy-intensity interval (HEAVY INT) exercise bout (n = 9). * indicates a significant difference from visit one. CFUs increased following the MOD INT (*p* = 0.045) and the HEAVY INT (*p* = 0.008) exercise bout.

5.4 Discussion

The population of postmenopausal women studied were healthy but presented risk factors for CVD. As a group, BMI was borderline overweight, total cholesterol and LDL were in the undesirable range and SBP was close to stage 1 hypertension [>140 mmHg, ACSM, (2006)]. Additionally, $\dot{V}O_{2max}$, a predictor of mortality (Laukkanen *et al.*, 2004) was lower than the young females in the previous chapter (young: 35 ± 5 ml·kg·min^{-1}, Postmenopausal: 22 ± 5 ml·kg·min^{-1}). Thus, these individuals would benefit from a lifestyle intervention such as exercise. The present study was the first to compare the acute effects of continuous and interval exercise, that were controlled for intensity, on vascular health in postmenopausal women. The main findings were that moderate-intensity (i.e. below the LT) continuous exercise had no immediate effect on endothelial function, carotid arterial stiffness and CAC number and

function. Conversely, in a sub-set of participants, moderate-intensity interval exercise acutely reduced carotid and brachial blood pressure and increased the ability of cultured CACs to form colonies *in vitro*. Additionally, work-matched interval exercise at a heavy-intensity also increased the number of CFUs, but appeared to elevate carotid and brachial blood pressure at 15 min post exercise.

5.4.1 Continuous and interval exercise did not acutely effect endothelial function

Moderate-intensity continuous and interval exercise and heavy-intensity interval exercise had no effect of brachial artery endothelial function in postmenopausal women. In response to lower-limb exercise, measurement of upper limb vascular function is used to reflect systemic endothelial function. Thus, the findings of the present study suggest that for increases in systemic endothelial function in postmenopausal women to occur, a greater stimulus or repeated acute exercise bouts are required. Conversely, an absolute increase of ~5% in brachial artery FMD was observed in a previous study observed following 45 min continuous treadmill exercise at 60% $\dot{V}O_{2max}$, in postmenopausal women with similar FMD values as participants in the present study [~5%, Harvey *et al*., (2005)]. Greater endothelial function following exercise is mediated by increases in nitric oxide bioavailability which induces vasodilation (Jungersten *et al.*, 1997; Rognmo *et al.*, 2008). During exercise, the endothelium experiences a greater magnitude of shear stress due to an increase in blood flow (Tinken *et al.*, 2010) that occurs in blood vessels supplying the working muscles and in the non-exercising limbs such as the brachial artery during cycling (Thijssen *et al.*, 2009a). Shear stress increases nitric oxide production following exercise through phosphorylation of eNOS by AMPK (Zhang *et al.*, 2006) or the PI3K/Akt pathway (Wang *et al.*, 2010). Therefore, given that the exercise duration was

greater in the study by Harvey *et al*., (2005) and a different definition of intensity adopted, participants may have experienced a greater shear stress and consequently enhanced nitric oxide bioavailability than the postmenopausal women in the present study, thus, explaining the discrepancy between these results. On the other hand, heavy-intensity interval exercise which would be expected to induce higher levels of shear stress, also had no impact on brachial artery FMD in the present study. This could be due to the reasons stated above or that blood pressure (present study; 137/84 mmHg vs. Harvey *et al*; 108/64 mmHg) and age (present study 64 ± 4 yrs vs. Harvey *et al*. 54 ± 2 yrs) were higher in postmenopausal women in the present study. Hypertension and older age (>60 years) have an additive effect on reducing nitric oxide bioavailability and increasing oxidative stress (Taddei *et al.*, 2001). Consequently, the postmenopausal women in the present study may require a higher level of absolute shear stress during exercise to counteract potential higher levels of oxidative stress and increase nitric oxide bioavailability. Additionally, the sensitivity of the endothelium to detect shear stress and trigger nitric oxide synthesis may have been reduced. Aged endothelial cells *in vitro* exhibit impaired eNOS protein upregulation in response to shear stress, when compared to young cells in the same culture conditions (Hoffmann *et al.*, 2001). Thus, a greater shear stress stimulus than younger women may be required to induce the same increases in nitric oxide. Therefore, an acute exercise bout of a higher relative intensity may be required to initiate short-term changes in brachial artery FMD in this older population of postmenopausal women.

5.4.2 Divergent effects of acute continuous and interval exercise on blood pressure

The postmenopausal women in the present study had greater (≥ 10 mmHg) carotid and brachial SBP, DBP, MAP and PP than the young women in the

previous chapter, placing individuals at a greater risk of CVD (Franklin *et al.*, 1999). Moderate-intensity continuous exercise did not reduce carotid or brachial artery blood pressure in postmenopausal women in the present study at 15 min post exercise. In contrast, brachial and carotid artery PP reduced following moderate-intensity interval exercise, whereas brachial artery SBP and PP and carotid artery SBP increased following heavy-intensity interval exercise. Thus, interval exercise appears to have a differential acute effect on blood pressure compared to continuous exercise.

Following moderate-intensity interval exercise, carotid and brachial artery PP reduced by 7 and 5 mmHg, respectively. This was caused by the reductions (although, not significant) in carotid and brachial SBP by 5 mmHg and 6 mmHg, respectively. These modest reductions in blood pressure have clinical importance as long term reductions in SBP by 5 mmHg decreases the risk of all cause mortality by 7% (Stamler *et al.*, 1989). Post-exercise hypotension is caused by reductions in systemic vascular resistance through alterations in neural control, such as reduced sympathetic nerve activity which decreases vasoconstriction, and by increases in vasodilatiors such as nitric oxide, prostaglandins, histamine and adenosine (Halliwill *et al.*, 2013). Conversely, in the present study, heavy-intensity interval exercise elevated brachial and carotid artery SBP, 15 min post-exercise. Given that exercise at a higher intensity produces greater oxidative stress (Goto *et al.*, 2003), and increasing age is associated with higher levels of oxidative stress (Taddei *et al.*, 2001), the elevated blood pressure post HEAVY INT exercise may be due to a lower bioavailability of vasodilators due to a greater level of ROS. Alternatively, hypotension might have been experienced post HEAVY INT at a later time point than 15 min post exercise due to the higher intensity of the exercise. However,

a time course post-exercise of blood pressure changes was not measured, which is a limitation of the study. Thus, it is not possible to determine whether MOD INT or HEAVY INT is better for acute alterations in blood pressure in postmenopausal women. It is important to note, that although reductions in PP post-MOD INT was observed in the subset of 9 participants; no changes were observed in the initial analysis between CON and MOD INT, when all 15 participants were included. A significant negative correlation between pre and delta MOD INT blood pressures (brachial artery PP, carotid artery PP and SBP) was only observed in the subset of 9 participants and not in the main group of 15 participants.

The absence of change in blood pressure post-CON exercise cannot be explained by either the exercise intensity or the time of the assessment (i.e. 15 min post-exercise), as interval exercise of the same intensity (moderate) measured at the same time point post-exercise, showed reductions in PP. This is in agreement with a previous study which reported hypotension (brachial artery SBP, MAP) at 15-30 min post-interval exercise, but not following post-continuous exercise, in healthy young men (Tordi *et al.*, 2010). The authors suggested that the fluctuations in cardiac output and thus, shear stress during interval exercise may have provided a greater stimulus for increases in vasodilators and the subsequent reduction in peripheral resistance. Thus, interval exercise might be a more potent stimulus than continuous exercise for reductions in blood pressure in postmenopausal women. However, future studies are required to examine the long-term effects of interval and continuous exercise training on blood pressure in postmenopausal women.

5.4.3 Carotid arterial stiffness was not acutely altered by continuous and interval exercise

Carotid arterial stiffness was higher in postmenopausal women than values reported from young women in the previous chapter and similar to values reported in a previous study in postmenopausal women (Moreau *et al.*, 2003). Acute changes in carotid arterial stiffness following a single bout of continuous and interval exercise have not been studied in postmenopausal women. The results of the present study observed no changes in any parameter of carotid arterial stiffness following any exercise bout. Acute changes in arterial compliance are related to functional alterations in vascular tone caused by changes in vasodilator bioavailability, especially nitric oxide (Sugawara *et al.*, 2007). Despite observing modest changes to carotid artery blood pressure, no change in endothelial function (a reflection of nitric oxide bioavailability) was observed, and likely explains why carotid arterial stiffness was unaltered. A potential explanation for no change in vascular tone relates to a low shear stress stimulus during exercise as mentioned previously. For carotid arterial stiffness to change, it is likely that repeated acute exercise bouts are required. Indeed, previous continuous exercise training studies in postmenopausal women have observed decreases in carotid arterial stiffness, which were suggested to be caused by reductions in the vasoconstrictor tone of vascular smooth muscle cells, and/or increases in the elastin and reductions in the collagen content of arterial walls (Moreau *et al.*, 2003; Sugawara *et al.*, 2006). Future studies are required to compare the effects of continuous and interval exercise training on carotid arterial stiffness in postmenopausal women.

5.4.4 Circulating angiogenic cells were not mobilisised following an acute bout of continuous and interval exercise

The present study was the first to compare CAC mobilisation following exercise in postmenopausal women. Furthermore, novel comparisons were made between interval and continuous exercise sessions. Although many studies have observed acute increases in CAC number following maximal and submaximal exercise in healthy and diseased populations (Adams *et al.*, 2004; Rehman *et al.*, 2004; Laufs *et al.*, 2005; Sandri *et al.*, 2005; Van Craenenbroeck *et al.*, 2008; Möbius-Winkler *et al.*, 2009; Cubbon *et al.*, 2010), a few have not (Shaffer *et al.*, 2006; Thijssen *et al.*, 2006; Van Craenenbroeck *et al.*, 2010). In agreement with the latter, this study also observed no increase in CACs following continuous and interval exercise at moderate and heavy-intensity. Potential explanations for these discrepancies might be explained by the population studied, the antibodies used to define a CAC, the volume and intensity of the exercise and the techniques used to analyse cell number (i.e. differences in the flow cytometer and gating strategies used). Acute exercise induced CAC mobilisation is mechanistically driven by an increase in shear-stress induced nitric oxide (Cubbon *et al.*, 2010). Thus, due to reasons previously mentioned such as an impaired endothelial sensitivity, a larger magnitude of shear stress might be required to mobilise CACs in postmenopausal women. This could be achieved by exercising at a higher intensity or by repeated acute bouts in a training programme.

5.4.5 Interval exercise acutely increases circulating angiogenic cell CFUs

The acute effect of exercise on CAC function has rarely been studied. The increase in CFUs following interval exercise regardless of intensity, but not following continuous exercise suggests that interval exercise is superior for

improving CAC function in postmenopausal women. However, this is potentially confounded by a trend (non-sig) for a reduction in CAC migration to VEGF following heavy-intensity interval exercise. An explanation for this discrepancy might be that the cells assessed in the migration and CFU assays are from two distinct populations. CACs cultured for 7 days that were used in the migration assay are believed to aid in endothelial repair through the secretion of cytokines and growth factors, and exhibit endothelial markers and characteristics (Rehman *et al.*, 2003; Hur *et al.*, 2004). In contrast, the CFUs are formed from the non-adherent cells collected on day 2 of culture, and although low numbers are associated with a greater risk of CVD (Hill *et al.*, 2003), the cells do not show endothelial characteristics such as tubule formation and are mainly monocytes (Rohde *et al.*, 2006). Indeed, it is suggested that CFUs are aggregated monocytes and T-cells (Rohde *et al.*, 2007) and reflect the interactive ability of the cells (Hirschi *et al.*, 2008). Given this, it is plausible that interval exercise produced a greater immune response than continuous exercise which contributed to a greater number of CFUs. Increases in immune cells following exercise are mediated by increased inflammation and damage induced during exercise (Pedersen & Hoffman-Goetz, 2000; Woods *et al.*, 2009). Thus, interval exercise of moderate and heavy-intensity might induce greater vascular/muscular stress than continuous exercise which increases the activity of immune cells that might aid in repair of vascular damage. Further investigation is warranted to validate this suggestion. Furthermore, greater damage/oxidative stress is produced at higher intensities (Goto *et al.*, 2003), which might explain the decrease in CAC migration to VEGF following heavy-intensity interval exercise. Evidence suggests that repeated exposure to acute exercise-induced inflammation is required for long term reductions in oxidative

stress and increased anti-oxidant activity (Gomes *et al.*, 2012). Thus, if postmenopausal women continue to perform interval exercise long term, greater vascular health benefits might be gained than that of continuous exercise. Nevertheless, if interval exercise induces greater acute vascular stress/immune response; the mechanisms for this remain unknown. Potentially, the fluctuations in cardiac output that result from the changes in work-rate associated with interval exercise have differential effects on the vasculature. Again, further investigations are required to discover these mechanisms.

5.4.6 Conclusions and future work

In conclusion, the government recommended guidelines of 30 min of moderate-intensity continuous exercise does not have an immediate impact on vascular health and repair in postmenopausal women. In contrast, interval exercise of a moderate and heavy-intensity is more potent for vascular changes. In postmenopausal women with a high blood pressure, moderate-intensity interval exercise might be better to induce reductions in SBP. For increases in nitric oxide for improvements in endothelial function and CAC number, a higher intensity, longer bouts and/or repeated exposure to shear is likely required for improvements in a population of postmenopausal women exhibiting CVD risk factors. Interval exercise of a moderate and heavy-intensity increases the colony-forming ability of cells potentially involved in the repair of vascular damage. Thus, investigations of the long-term impact this specific type of interval exercise has on vascular health and repair are warranted in postmenopausal women and other populations that are at risk of developing CVD, such as obesity. Additionally, as moderate-intensity continuous exercise had no immediate impact on vascular health and repair in postmenopausal women, heavy-intensity continuous exercise should be investigated.

5.4.7 Study limitations

The techniques used to measure CAC cell number and function may have limited the ability to detect changes to these variables following exercise due the exclusion of many participants. The flow cytometer failure occurred several times which significantly reduced the number of participants that could be included in the CAC number analysis. This led to the purchase of a new flow cytometer which was used for subsequent studies. In a few participants the migration and adhesion CAC function analysis could not be completed because not enough cells were collected on day 7 of culture. In future studies the cell culture was adjusted so that more cells would survive culture, in addition to cell culture characteristics measured to identify differences from pre to post-training (refer to section 3.12.1 in the general methods, chapter 3). Furthermore, different techniques for assessing CAC function were piloted, which involved a lower number of cells on day 7 of culture, to resolve the problem of too few cells harvested at the end of culture. The CAC migration assay in the present and previous chapter had specific limitations. Firstly, the reported values in the literature (Laufs *et al.*, 2005) are higher than those reported in populations of young and postmenopausal women in the present and previous chapters. Additionally, in a few participants more cells migrated to the control stimuli rather than VEGF, which has either not been observed or not discussed in previous studies. Furthermore, for both the migration and adhesion assays, the analysis was conducted following a 24 hr incubation period after 7 days of culture. Thus, short term changes were not examined. Future studies should examine a time course of changes in CAC function from day 7 of culture to +24 hr post. Consequently, in the next chapter as discussed in section 3.12.5 of the

general methods, chapter 3, a new method to assess CAC function was developed which involved a time course of change.

The interval exercise bouts in the present study were modified from the original study by Turner *et al*., (2006) as discussed. However, blood lactate and $\dot{V}O_2$ were not recorded during the sessions, therefore the exercise intensity of the moderate and heavy-intensity exercise bouts could not be confirmed. The addition of these measures during exercise sessions should be included in future exercise studies to confirm that the exercise is in the correct intensity domain.

Chapter 6 The impact of heavy-intensity interval and continuous exercise training on vascular health and repair in obese/overweight premenopausal women

Aspects from this chapter were presented at the following conferences:

- European College of Sports Science, Annual meeting, Barcelona, Spain, June 2013.
- American Physiological Society Intersociety Meeting: The Integrative Biology of Exercise VI, Westminster, CO, October 2012.

6.1 Introduction

Obesity is a major health burden, responsible for 5% of all global deaths (World Health Organisation, 2011) which significantly increases the risk of developing CVD. Worldwide, 35% of women are overweight and 13.8% are obese (World Health Organisation, 2011). Overweight/obese (BMI > 25.0 $kg \cdot m^{-2}$) adults exhibit an impaired endothelial function (Perticone *et al.*, 2001), increased inflammatory cytokines, adhesion molecules and oxidative stress (Ziccardi *et al.*, 2002; Furukawa *et al.*, 2004), reduced number and function of CACs (Muller-Ehmsen *et al.*, 2008; Heida *et al.*, 2010), and augmented arterial stiffness (Zebekakis *et al.*, 2005). Lifestyle interventions such as exercise can reduce the risk of CVD in obesity by ameliorating the changes in the above variables. Given that the prevalence of obesity is growing with an estimated 11 million more adults in the UK (increase from 26% to 35-43% in women) to suffer from the condition from 2010 to 2030 (Wang *et al.*, 2011), it is especially important to identify the most effective exercise that will yield the greatest benefits to vascular health and repair.

The American Heart Association (AHA) recommend that for overweight/obese women who need to lose weight, a minimum of 60-90 min of moderate-intensity exercise should be achieved on all days of the week (Mosca *et al.*, 2011). However, vascular health can be improved without weight loss. In mice following exercise training without reductions in weight, an increased endothelial-dependent vasodilation, nitric oxide bioavailability, superoxide dismutase, total and phosphorylated eNOS and reduced oxidative stress was reported (Moien-afshari *et al.*, 2008). Similarly, in overweight/obese adults following exercise training without changes in BMI, inflammatory cytokines and endothelial-dependent vasodilation increased, and systolic blood pressure reduced (Dekker *et al.*, 2007; Kadoglou *et al.*, 2007; Mestek *et al.*, 2010). Furthermore, reductions in arterial stiffness and increases in CAC number have been observed following exercise training in obese adults with only small reductions in BMI (~1 $kg \cdot m^{-2}$ (Cesari *et al.*, 2012; McNeilly *et al.*, 2012)]. Additionally, the AHA recommendations are impracticable for many, given that the greatest barrier to exercise in women is a lack of time, as mentioned in chapter 4. Time-efficient methods of exercise such as sprint training as seen in previous studies (Burgomaster *et al.*, 2008; Rakobowchuk *et al.*, 2008) and in chapter 4, increased cardio-respiratory fitness, endothelial function and CAC number in healthy adults. In obese sedentary men, 2 weeks of SIT improved $\dot{V}O_{2max}$, insulin sensitivity and reduced systolic blood pressure (Whyte *et al.*, 2010). However, this type of exercise requires strong motivation and is often accompanied by feelings of nausea. Thus, this method of training may likely be unenjoyable in sedentary overweight/obese women, leading to reduced exercise adherence.

The effects of alternative interval exercise protocols involving short work:recovery duty cycles on vascular health and repair have recently been investigated. In coronary artery disease patients, 12 weeks of interval exercise involving 60 s: 60 s work:recovery duty cycles improved brachial artery FMD to a similar extent as continuous exercise of a greater volume (Currie *et al.*, 2013). Additionally, an acute bout of interval exercise involving 1:2 work:recovery duty cycles in postmenopausal women in the previous chapter (section 5.3.5.3) increased CAC colony forming units, whereas continuous exercise had no acute effect on vascular health and repair. Moreover, moderate and heavy-intensity interval exercise training involving a 1:2 work:recovery duty cycle ratio, improved endothelial reactivity to low shear stress in healthy individuals (Rakobowchuk *et al.*, 2012). However, the effects of this type of interval exercise on vascular health and repair has not been assessed in obese women.

Although interval exercise has been shown to improve elements of vascular health to a similar or greater extent than continuous exercise; the intensity of the exercise groups have not been appropriately matched in previous studies. In obese patients with the metabolic syndrome, 16 weeks of interval exercise involving 4 x 4 min periods at 90% HR_{max} separated by 3 min recovery at 70% HR_{max}, increased nitric oxide and endothelial function to a greater extent than continuous exercise at 70% HR_{max} (Tjønna *et al.*, 2008). As explained in the previous chapter, prescribing exercise using a percentage of HR_{max} does not take into account individual differences in the LT, and thus, the intensity of the exercise cannot be defined. In the previous chapter exercise intensity was defined according to exercise intensity domains. An acute bout of heavy-intensity interval exercise was compared with moderate-intensity continuous and interval exercise in postmenopausal women, with results suggesting that

moderate-intensity continuous exercise had no immediate effect on vascular health and repair, and may not be as effective for ameliorating CVD risk factors as interval exercise. However, heavy-intensity interval exercise has not been compared to heavy-intensity continuous exercise. Therefore, the aims of this chapter were to compare heavy-intensity interval exercise training involving short work:recovery duty cycles, with work-matched heavy-intensity continuous exercise training on vascular health and repair in overweight/obese women.

6.2 Methods

6.2.1 Participants

Participants were recruited through poster and email advertisements placed around the local area and by contacting participants from a previous study who had given consent to be contacted in the future for further studies. Twenty healthy overweight/obese women (age: 42 ± 6 yrs) volunteered for the study. Participants were required to have a BMI ≥27 $kg \cdot m^{-2}$ and/or a WHR >0.8. Exclusion criteria (chapter 3, section 3.1) were confirmed prior to participation through a telephone interview.

6.2.2 Experimental protocol

Participants attended the University of Leeds laboratory on two occasions prior to the completion of a 12 week exercise training programme. On visit one, vascular measures and a fasted blood sample (60 ml) for the assessment of blood plasma inflammatory markers and CAC number and function were completed. On the second visit a cardio-respiratory fitness test was completed for the assessment of maximal aerobic capacity ($\dot{V}O_{2max}$), the LT and to enable calculation of the work-rates in the exercise sessions. Control factors for these two sessions are specified in the general methods, chapter 3, section 3.2. Following the exercise intervention, the vascular and blood assessments were

completed between 48-72 hrs after the last training session and the cardio-respiratory fitness test completed within one week. After completion of the pre-training assessments, participants were matched for age and BMI and assigned to either a heavy-intensity interval (INT, n = 10) or heavy-intensity continuous (CON, n = 10) exercise training group. Exercise sessions were supervised at the University of Leeds exercise physiology laboratory twice per week with one unsupervised exercise session performed at home. After ~4 weeks participants attended the laboratory to assess short term functional changes in brachial artery endothelial function. At mid-point in the exercise training programme (6 weeks), participants completed a cardio-respiratory fitness test instead of a training session to assess changes in $\dot{V}O_{2max}$ and the LT. If necessary, exercise session work-rates were adjusted if increases in the $\dot{V}O_{2max}$ and the LT were observed. To control for hormonal influences, participants who were not taking any hormonal contraception completed the vascular and blood assessments between day 1-10 of the menstrual cycle. However, one participant was assessed during the late luteal phase at all time points due to scheduling constraints. Individuals who were taking the combined contraceptive pill were assessed between day 1-7 of the “pill free” week. Thus, participants were assessed when oestrogen levels were at the lowest. For participants who were taking progesterone only contraception and had an absence of menses, the vascular and blood assessments were acquired at any time.

6.2.3 Variables assessed pre, mid and post-training

The protocols for the following variables are described in detail in the general methods chapter (chapter 3).

6.2.3.1 Cardio-respiratory fitness

At pre, mid and post-training a seated ramp-incremental step exercise test (RISE-105) was performed for the assessment of $\dot{V}O_{2max}$, LT, RI test duration, WR_{peak} (section 3.3) and for calculation of the work-rates achieved at these points for the subsequent exercise sessions. The ramp rate of the RI stage of the test was 12 W/min and the step exercise work-rate set at 105% WR_{peak}. Due to equipment failure, the SE test was not completed in one participant at pre-training, one participant at mid-training and two participants at post-training. In these participants, the $\dot{V}O_{2peak}$ value from the RI stage of the test was reported as $\dot{V}O_{2max}$. WR_{LT} and the exercise session work-rates were determined independently by two researchers and an average calculated.

6.2.3.2 Vascular measures

The following vascular measures were completed at pre and post-exercise (sections 3.4-3.8): BMI, WHR, resting HR, brachial artery FMD, peak reactive hyperaemia, peak shear rate, AUC_{peak}, AUC_{60} and AUC_{90} and their corresponding VTIs, brachial artery blood pressure, PWV_{cr}, PWV_{bf}, and carotid arterial blood pressure, cross-sectional compliance (CSC), distensibility, β-stiffness index and IMT. During recording of brachial artery blood velocity, the Doppler insonation angle for each participant between pre and post-testing was within 3°. Brachial artery endothelial function was also assessed at ~4 weeks from the start of training, following the same protocol as pre and post-training.

6.2.3.3 Blood markers and CAC number and function

At pre and post-training a 60 ml fasted blood sample was taken following the procedure outlined in section 3.9 and collected in EDTA vacutainers and a sodium citrate tube (for SDF-1α analysis) for plasma inflammatory markers and CAC number and function analysis. Blood plasma was separated and analysed

for TNF-α, IL-6, hsCRP, sVCAM-1, VEGF and SDF-1α (assessed from platelet free plasma) using commercially available ELISA kits (section 3.10) for the assessment of inflammatory makers and cytokines involved in CAC mobilisation. For flow cytometry CAC enumeration, 10 ml of blood was used to quantify the number of $CD34^+$, $CD34^+CD45^{dim}$ and $CD34^+CD45^{dim}KDR^+$ CACs following a modified ISHAGE protocol described in section 3.11.2.3. The number of leukocytes within this sample was counted using a haemocytometer and reported per ml of blood. The remaining 30 ml blood sample was used for functional assessment of CACs (section 3.12). Briefly, mononuclear cells were separated from the blood, plated on fibronectin own-coated wells and cultured for 7 days. Cell growth characteristics were determined on days 2, 4 and 7 of culture. The secretion of VEGF, SDF-1α and G-CSF from CACs was measured from conditioned medium using commercially available ELISA kits for assessment of paracrine function. The function of CACs cultured for 7 days was assessed by their ability to adhere to a monolayer of saphenous vein VSMCs *in vitro*. The VSMCs were collected from non-diabetic male patients (age: 54-68 yrs) undergoing coronary artery bypass graft surgery. For each participant at pre and post-testing, the CAC adhesion assay was performed on VSMCs collected from the same patient.

6.2.4 Exercise training protocol

Participants completed 2 supervised training sessions per week in the laboratory for a 12-week period. All sessions were performed on a cycle ergometer (Lode BV, Excalibur Sport V2.0, the Netherlands). An unsupervised exercise session was performed weekly. In this session, participants were instructed to complete one 30 min brisk walk each week. All participants exercised in the heavy-intensity domain regardless of exercise type, and groups

were matched for work. To confirm that participants were within the correct exercise intensity domain, during the first training session breath by breath data was collected for analysis of $\dot{V}O_2$ and fingertip capillary blood samples were acquired using an automated blood lactate analyser (Lactate Pro, Arkray, Japan) to determine blood lactate concentrations. At rest prior to the exercise session, two blood lactate samples were obtained at 5 min intervals and an average calculated. For the INT group, blood lactate was taken every 5 min in the first training session. As CON exercise sessions were shorter than the INT exercise sessions, because groups were matched for work (section 6.2.4.2), blood lactate was taken every 4 min on the 3rd-6th training session when the duration of the session was ≥16 min.

6.2.4.1 Interval exercise sessions

Interval exercise sessions were based on the 1:2 (work:recovery) duty cycles first described by Turner et al., (2006). The duty cycles and their corresponding intensity domains are discussed in chapter 5, section 5.2.4 (Figure 5.2). In the previous chapter (chapter 5), the duty cycle work-rates were adjusted to be suitable for the population of postmenopausal women. In the present chapter, the duty cycles were modified further and involved 40 s at 70% delta work-rate followed by 80 s active recovery at 20 W. 70% delta (Δ) is 70% of the difference between WR_{peak} and WR_{LT} calculated as:

$$70\%\Delta = 0.7\left(WR_{peak} - WR_{LT}\right) + WR_{LT}$$

This work-rate was chosen instead of normalising to a percentage of $\dot{V}O_{2max}$/ WR_{peak} as Δ takes into account individual differences in the LT, whereas scaling to a percentage of $\dot{V}O_{2max}$ can place individuals in different intensity domains (i.e. above or below the LT). For example, as demonstrated

by Rossiter, (2011) in two participants performing constant load exercise (30 min) at the same work-rate and the same percentage of $\dot{V}O_{2max}$ (85%), one participant could not complete the session due to attainment of $\dot{V}O_{2max}$. However, the other participant completed the entire session with $\dot{V}O_2$ attaining a steady-state. Thus, normalising to a percentage of $\dot{V}O_{2max}$ to define exercise intensity can result in participants experiencing different physiological stresses. The work-rate of 70%Δ was chosen as it is above critical power (i.e. the highest constant load work-rate that can be attained whilst remaining at a steady-state) and thus, cannot be sustained when performed continuously (Cannon *et al.*, 2011). INT exercise duty cycles at this work-rate were piloted in one participant. Initially, a 30:60 s duty cycle was chosen as this has been shown to be characteristic of heavy-intensity exercise (Turner *et al.*, 2006). As the work-rate was modified from the original study, a further session was completed involving a longer duty cycle of 40:80 s. Breath by breath data was collected throughout the session for analysis of $\dot{V}O_2$. Both sessions were characteristic of heavy-intensity interval exercise as the peaks of the $\dot{V}O_2$ profile were above the LT and remained at that level throughout (Figure 6.1). Therefore, the 40:80 s duty cycle was chosen as the $\dot{V}O_2$-time integral (21051 ml) and the average $\dot{V}O_2$ (1076 ml·min^{-1}) was slightly greater than the 30:60 s session (20260 ml and 1034 ml·min^{-1}, respectively), and thus, might provide a greater stimulus for physiological adaptations.

For all participants in the INT exercise group, session duration in week one was 20 min, which increased to 25 min in week 2, 30 min in week 3, 35 min in week 7 and 40 min in week 10.

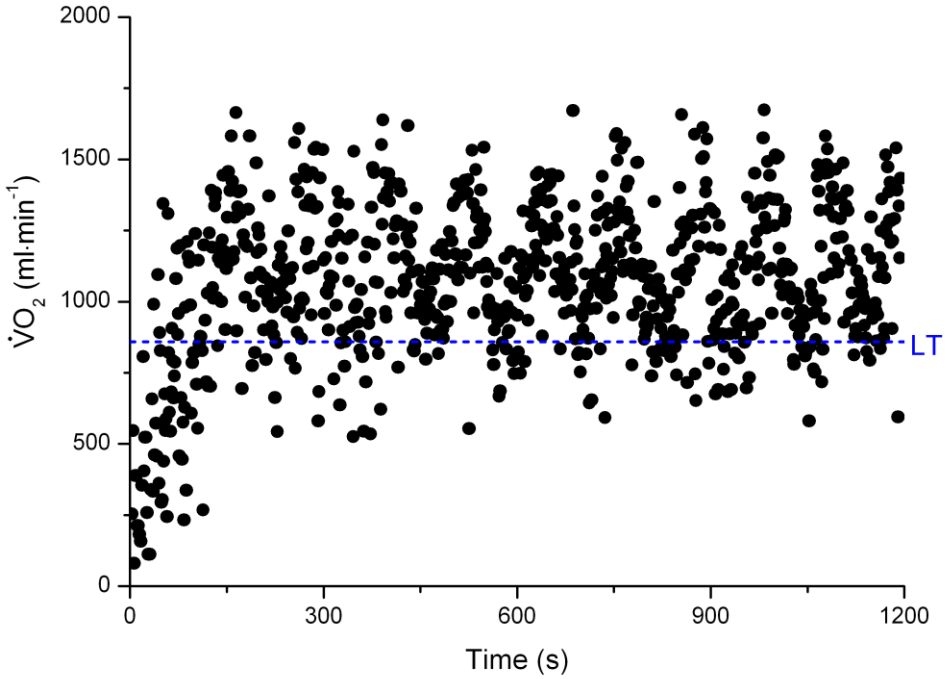

Figure 6.1. The $\dot{V}O_2$ profile during a INT exercise session involving 40:80 s duty cycles in a pilot participant. The peaks of the $\dot{V}O_2$ oscillations are above the individual lactate threshold (LT, blue dashed line, 859 ml·min^{-1}) and remains stable, thus, in the heavy-intensity domain.

6.2.4.2 Continuous exercise sessions

The CON exercise sessions involved cycling at 20%Δ work-rate for the duration of the session. 20%Δ was chosen so that participants would be exercising above their individual LT but below critical power and thus, in the heavy-intensity domain (Cannon *et al.*, 2011). To enable participants in the CON exercise group to complete the same amount of work (kJ) as the participants in the INT exercise group, the amount of work that would be completed if the CON participants completed an INT exercise session was calculated. This was calculated for each INT session duration (i.e. 20, 25, 30, 35, 40 min). Subsequently, the CON duration for each session was calculated using the following equation:

$$Time = \frac{work}{power}$$

Where $work$ (J) is the amount completed in an INT exercise session involving 40 s at 70%Δ work-rate and 80 s at 20 W, and $power$ (J/s) is the CON exercise session work-rate of 20%Δ. The group average CON exercise session durations for weeks 1, 2, 3-6, 7-9 and 10-12 were 13.3 ± 1.3 min, 16.6 ± 1.7 min, 19.9 ± 2.0 min, 22.5 ± 2.1 min and 25.8 ± 2.4 min, respectively. Thus, exercise groups were matched for exercise intensity domain (heavy) and for work done, but differed in regards to session duration and the work-rate profile (interval vs. continuous). Figure 6.2 shows a schematic representation of the INT and CON exercise sessions in week one.

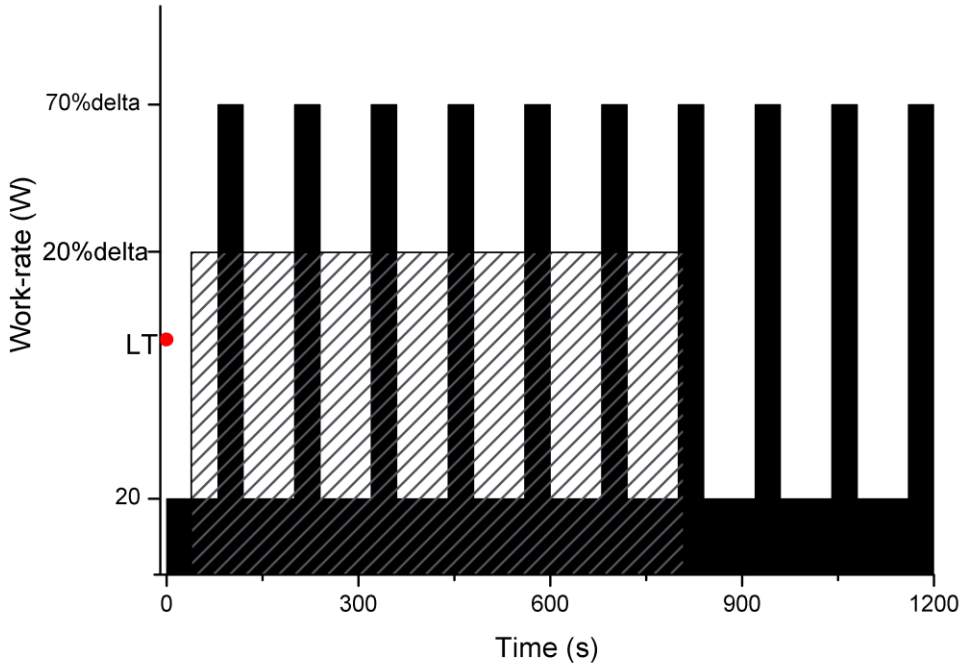

Figure 6.2. Schematic of the heavy-intensity interval (INT) and continuous (CON) exercise training sessions in week one. INT exercise sessions (solid black bars) involved 40:80 s duty cycles at 70%delta work-rate (ave: 124 ± 25 W) followed by 20 W recovery for 20 min duration. Work-matched CON exercise sessions (striped gray area) involved a continuous work-rate (ave: 85 ± 16W) which finished when the participant completed the same amount of work which would be achieved in an INT exercise session. Average CON duration in week 1 was 13.3 ± 1.3 min. The work-rates for both groups were above the lactate threshold (LT, red circle) work-rate (WR_{LT} ave: 62 ± 15 W).

6.2.5 Statistical analysis

The variable “time from cuff release to peak diameter” was not normally distributed therefore, the positive skew was corrected using reciprocal transformation of the variable (1/x). CAC number was also not normally distributed. Therefore, to correct for the positive skew, $CD34^+$ and $CD34^+CD45^{dim}$ CACs were natural log (lnx) transformed and $CD34^+CD45^{dim}KDR^+$ CACs square root transformed (√x), as lnx cannot be applied to zero values. The effect of the training interventions was analysed using a mixed mode repeated measures ANOVA with time (pre vs. post-training) as the within-subjects factor and training group (INT vs. CON) as the between-subjects factor. For the assessment of endothelial function and cardio-respiratory fitness, mid-training was added as a time factor to the ANOVA. At pre-training there were significantly lower numbers of $CD34^+CD45^{dim}$ CACs in the INT training group when compared to the CON group (p = 0.01). Therefore, pre-training values of $CD34^+CD45^{dim}$ CACs were added as a covariate to the ANOVA. Paired t-tests were conducted for post-hoc analysis to identify which exercise bout showed a significant change. The percentage change in CAC adhesion from pre to post-training was analysed for group differences via a Mann-Whitney test, as data could not be normally distributed using transformations. Exercise session characteristics and the percentage change from pre to post-training in cell area were assessed for training group differences via a Student’s independent t-test. To assess the changes in blood lactate levels between rest and each time point during the first exercise session, a repeated measures ANOVA was performed with time as the within-subjects factor. Following continuous exercise training in overweight/obese adults, previous studies reported increases in brachial artery FMD from 2% to 5%

(Lavrenčič *et al.*, 2000; Tjønna *et al.*, 2008; Stensvold *et al.*, 2010). Therefore using an increase of 4% in brachial artery FMD and a standard deviation of 3% (reported from healthy women in chapter 4), a minimum of 20 participants in total were required to obtain 80% power (α = 0.05) in a two-treatment parallel-design study.

6.3 Results

6.3.1 Exercise session characteristics

In previous studies CON exercise sessions have been matched for intensity with INT exercise sessions using the INT session average percentage of $\dot{V}O_{2max}$ (Tjønna *et al.*, 2008; Tordi *et al.*, 2010; Bartlett *et al.*, 2012). In this study the average within exercise $\dot{V}O_2$ from the INT exercise group was 1036 ml·min^{-1} which equated to ~50% of $\dot{V}O_{2max}$. If participants in the CON exercise group were to exercise at a constant work-rate equivalent to 50% of $\dot{V}O_{2max}$, some participants would be above and others below their individual LT (Figure 6.3). Thus, participants would be exercising in different intensity domains and would not be matched to the INT exercise sessions in the heavy-intensity domain.

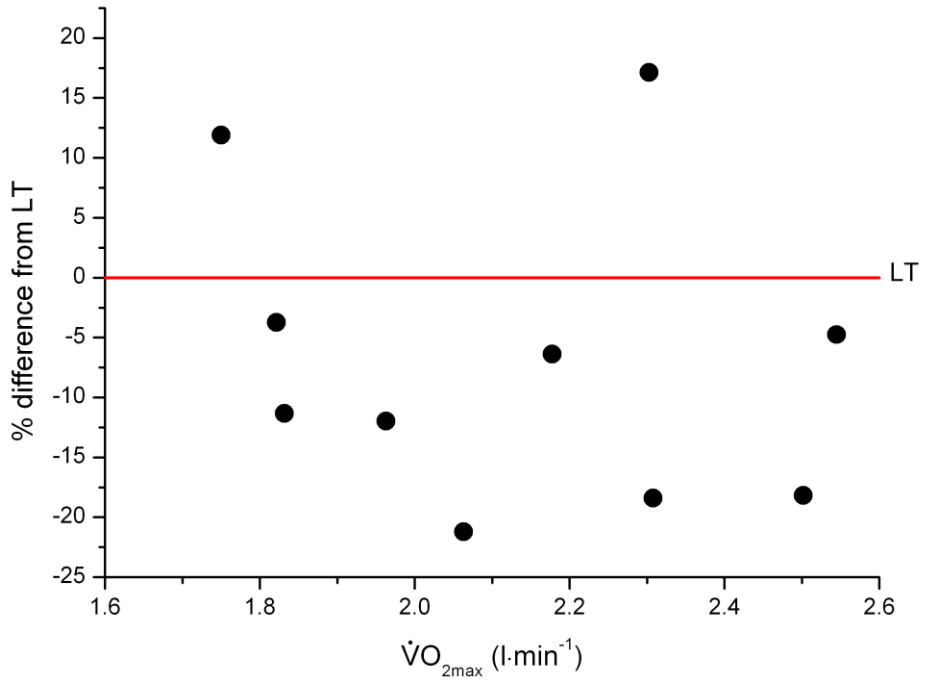

Figure 6.3. The percentage difference from the lactate threshold (LT) if participants in the CON exercise group were to exercise at the average percentage of $\dot{V}O_{2max}$ from the INT exercise group (50% $\dot{V}O_{2max}$). If participants in the CON exercise group were working at 50% of $\dot{V}O_{2max}$, two participants would be above their individual LT and in the heavy-intensity domain where as the remaining participants would be below their LT and in the moderate-intensity domain.

Therefore, in the present study INT and CON exercise groups were matched to the heavy-intensity domain and not scaled to a fixed percentage of $\dot{V}O_{2max}$. The group average characteristics of the first exercise session are displayed in Table 6.1. Work completed and average HR did not differ significantly between groups (p > 0.05). Average $\dot{V}O_2$ was significantly greater in the CON exercise group when compared with the INT exercise group (p < 0.01, Table 6.1). However, both the INT and CON exercise sessions were confirmed to be of a heavy-intensity as $\dot{V}O_2$ during the CON session increased to above the individual LT and attained a steady-state (Rossiter, 2011), and the peaks of the $\dot{V}O_2$ profile during the INT session also increased to above the LT and

remained at a stable level [Figure 6.5; Turner *et al*., (2006)]. Additionally, blood lactate levels were significantly elevated above resting values at each time point during the exercise session ($p < 0.001$, Figure 6.4), and plateaued in both groups (blood lactate levels between the final 3 time points during the exercise session were not significantly different in both groups, $p > 0.05$), which is characteristic of heavy-intensity exercise (Rossiter, 2011).

Table 6.1. The average (mean ± SD) exercise session characteristics during the first interval (INT) and continuous (CON) exercise training sessions.

	INT	**CON**
WR_{LT} (W)	57 ± 15	68 ± 14
70%Δ WR (W)	124 ± 25	127 ± 23
20%Δ WR (W)	n/a	85 ± 16
Average work/session (kJ)	66 ± 10	67 ± 9
Average $\dot{V}O_2$/session ($ml \cdot min^{-1}$)	1036 ± 112	1331 ± 198 *****
Average HR/session (bpm)	121 ± 17	132 ± 5

***** indicates a significant difference between the INT training group ($p < 0.01$). WR_{LT} = work-rate at the lactate threshold, ΔWR = difference between the work-rate at the LT and $\dot{V}O_{2max}$, HR = heart rate and bpm = beats per minute.

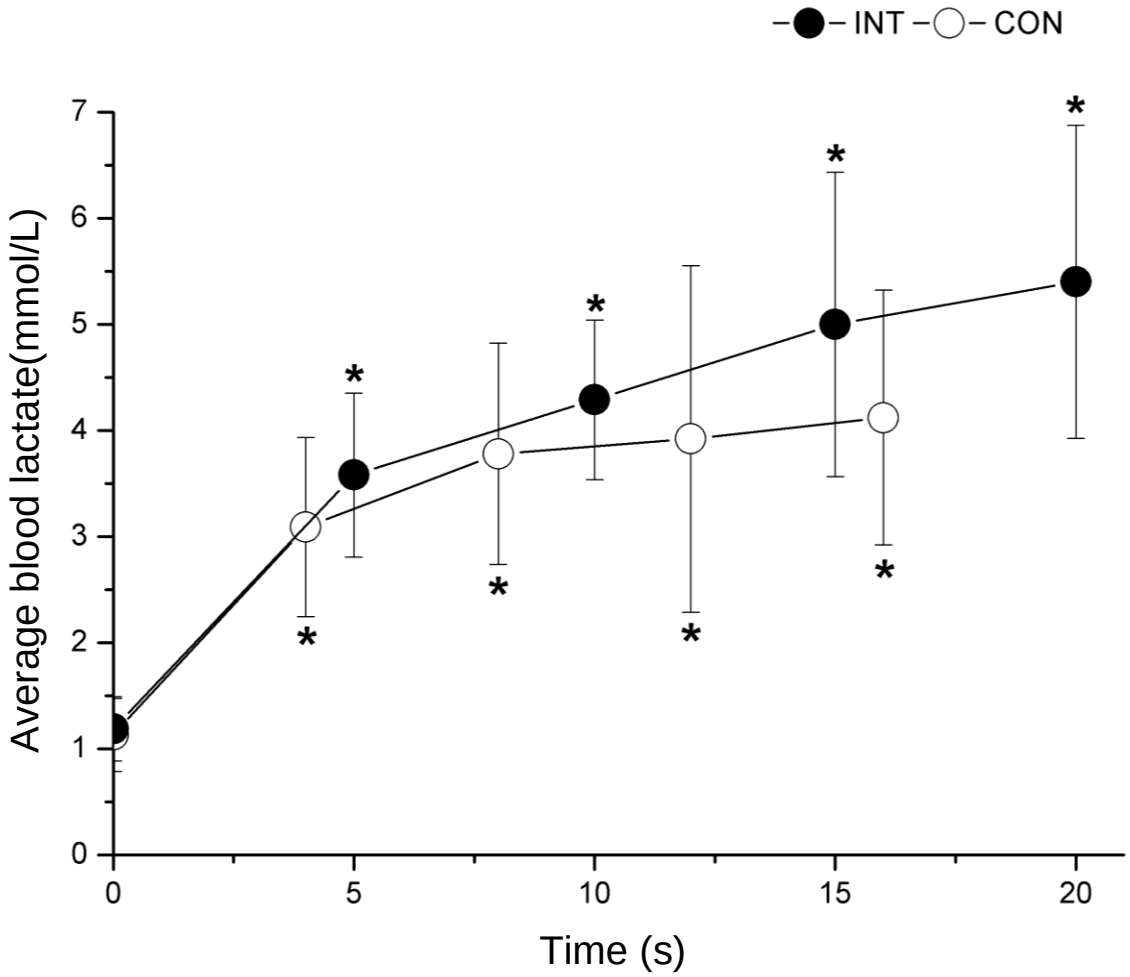

Figure 6.4. Group average (± SD) blood lactate response to interval (INT) and continuous (CON) exercise. * indicates a significant difference from rest ($p < 0.05$). Blood lactate levels were not significantly different between the two final time points in both exercise groups.

Figure 6.5 displays the group average $\dot{V}O_2$ response to the first INT and CON exercise training session. The group average area under the $\dot{V}O_2$ - time curve for the first training session was greater in the INT exercise group (20175 ml) when compared to the CON exercise group (17120 ml).

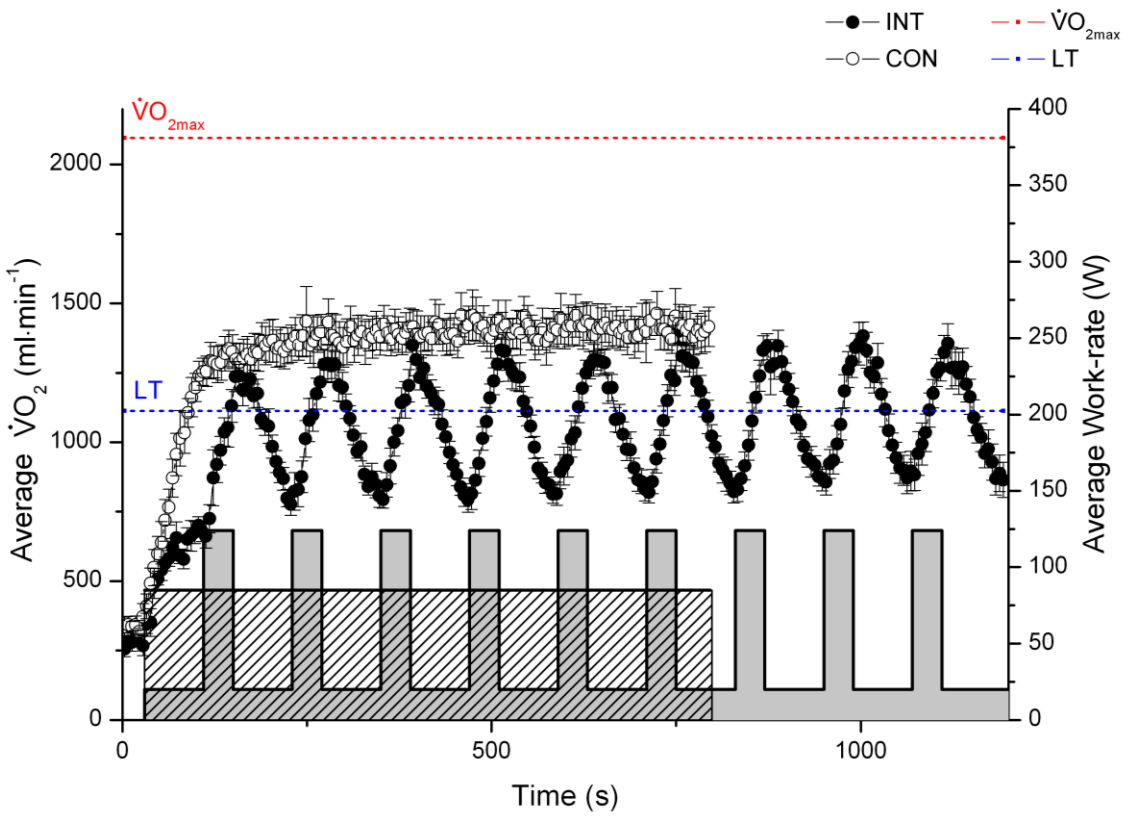

Figure 6.5. Group average (± SD) $\dot{V}O_2$ response to interval (INT, closed circles, shaded grey work-rate profile) and continuous (CON, open circles, striped work-rate profile) exercise in training session one. The red dashed line represents group average $\dot{V}O_{2max}$ (2096 ± 337 ml·min^{-1}) and the blue dashed line represents group average lactate threshold (LT, 1113 ± 208 ml·min^{-1}).

Figure 6.6 displays the group average HR response to the first INT and CON exercise training session. The group average area under the HR - time curve for the first training session was greater in the INT exercise group (2417 beats) when compared to the CON exercise group (1726 beats). On average, participants in the INT exercise group were cycling at a lower average percentage of HR_{max} (67 %) whereas the CON exercise group were cycling at 75% of HR_{max}.

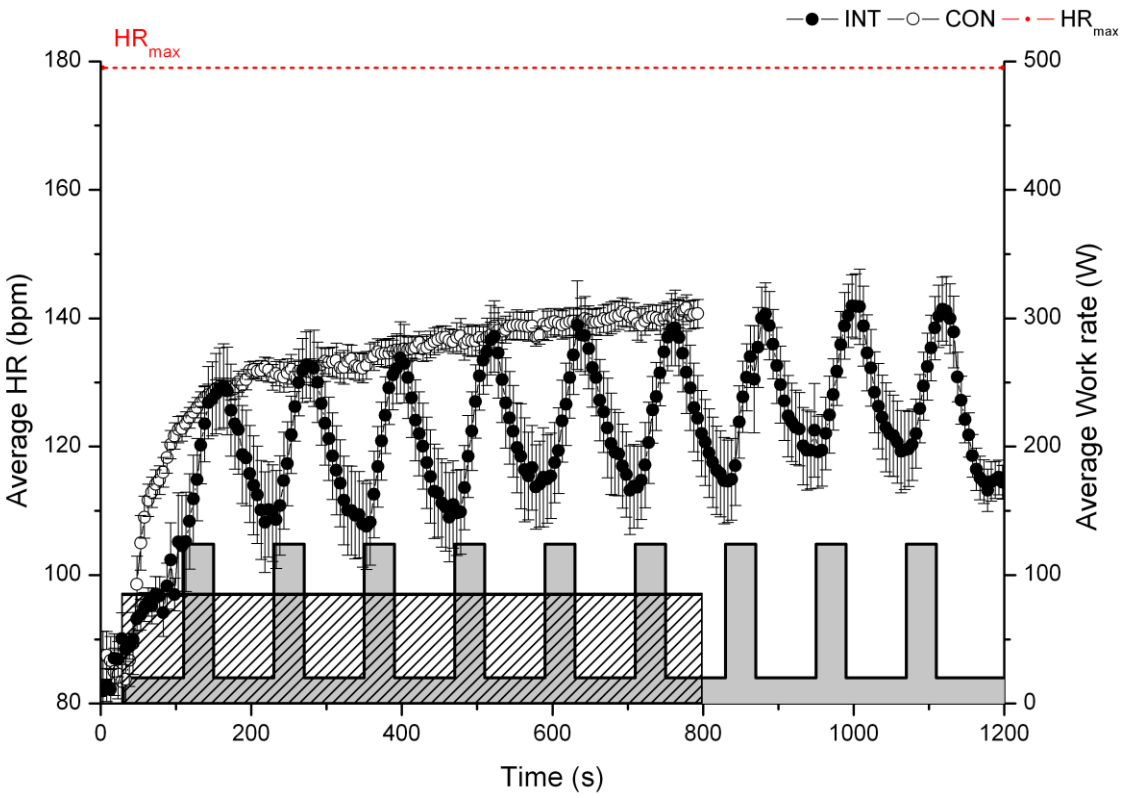

Figure 6.6. Group average (± SD) heart rate (HR) response to interval (INT, closed circles, shaded grey work-rate profile) and continuous (CON, open circles, striped work-rate profile) exercise in training session one. The red dashed line represents group average maximal heart rate (HR_{max}, 179 ± 9 bpm).

6.3.2 Participant characteristics

One participant from the INT exercise group completed only 10 weeks of training due to injury, which was not related to the exercise training programme. Also, one participant from the CON exercise group completed 10 weeks of training due to holiday, but the duration of the sessions were increased to enable the participant to complete the correct amount of total work that would have been achieved if she had completed the total 12 weeks. All other participants completed the full training programme. Participant demographics (mean ± SD) are displayed in Table 6.2. There was no effect of the training regardless of exercise group on BMI, WHR, resting HR and brachial artery DBP, MAP and PP. However, brachial artery SBP showed a trend for a reduction following training in both groups (time effect p = 0.08, time by group

interaction $p > 0.05$). The 95% CIs for the pre to post-training difference in SBP for the INT and CON group were -12 to +0.4 mmHg and -5 to +4 mmHg, respectively (Figure 6.7a). Participants with a higher pre-training SBP gained a greater reduction in SBP following training (r = -0.47, $p = 0.04$). However, when the correlation analysis was performed on the individual groups, only the INT group showed a significant negative correlation between pre-training and delta SBP (INT: r = -0.77, $p = 0.01$; CON: r = -0.29, $p = 0.41$, Figure 6.7b-c).

Blood plasma was analysed from 17 participants (INT n = 7, CON n = 10) due to difficulties in blood collection and 2 participants who did not want to give blood. The levels of TNF-α were undetectable in the plasma using the ELISA kit in all participants and therefore analysis was not performed on this variable. There were no observed changes in plasma concentrations of IL-6 ($p = 0.84$), hsCRP ($p = 0.54$) and sVCAM-1 ($p = 0.42$) with no time by group interactions ($p > 0.05$, Table 6.2).

Table 6.2. Participant demographics (mean ± SD) at pre and post 12 weeks of either interval (INT) or continuous (CON) exercise training.

	INT (n = 10)		CON (n = 10)	
	Pre	**Post**	**Pre**	**Post**
BMI ($kg \cdot m^{-2}$)	32.7 ± 3.6	32.0 ± 3.6	31.3 ± 3.7	31.0 ± 3.5
WHR (a.u.)	0.90 ± 0.1	0.89 ± 0.1	0.90 ± 0.1	0.90 ± 0.1
Resting HR (bpm)	68 ± 6	68 ± 11	66 ± 10	68 ± 7
Brachial artery SBP (mmHg)	124 ± 11	119 ± 7	125 ± 15	124 ± 15
Brachial artery DBP (mmHg)	81 ± 9	79 ± 8	82 ± 10	82 ± 10
Brachial artery MAP (mmHg)	96 ± 10	92 ± 8	96 ± 11	96 ± 11
Brachial artery PP (mmHg)	43 ± 5	40 ± 3	43 ± 11	42 ± 8
Plasma IL-6 (pg/ml)	3.2 ± 0.8	3.4 ± 1.5	3.6 ± 1.0	3.5 ± 0.9
Plasma hsCRP (µg/ml)	1.9 ± 1.3	1.7 ± 1.2	1.9 ± 1.3	1.8 ± 1.9
Plasma sVCAM-1 (ng/ml)	511 ± 56	502 ± 52	512 ± 80	503 ± 61

BMI = body mass index, WHR = waist to hip ratio, HR = heart rate, SBP = systolic blood pressure, DBP = diastolic blood pressure, MAP = mean arterial pressure, PP = pulse pressure, IL-6 = interleukin-6, hsCRP = high sensitivity C-reactive protein and sVCAM-1 = soluble vascular cell adhesion molecule-1.

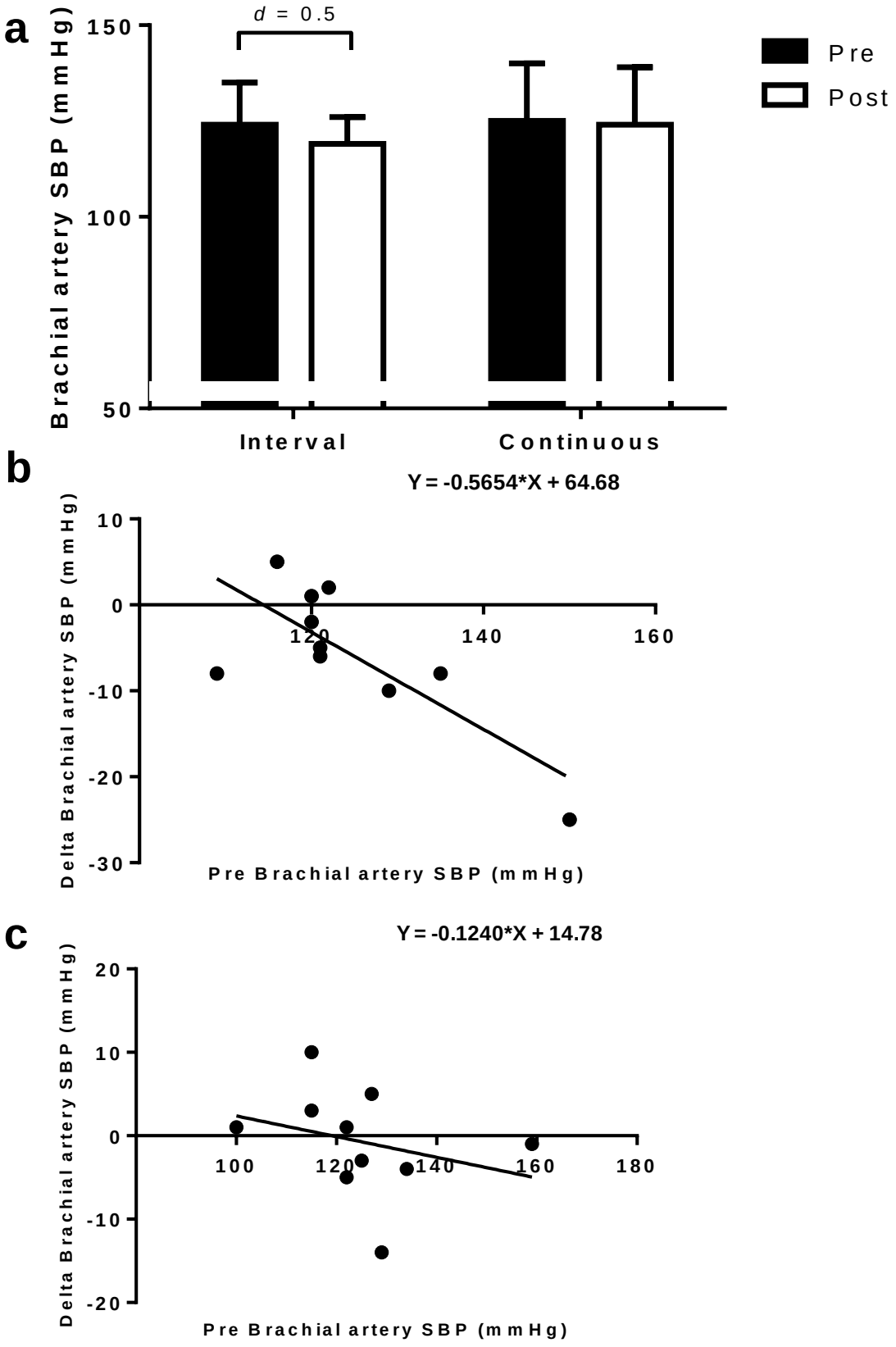

Figure 6.7. Changes in brachial artery systolic blood pressure (SBP) with exercise training. A medium effect size (*d* = 0.5) for a decrease in SBP following interval exercise was observed (a). Participants in the interval training group with a higher pre-training SBP exhibited a larger decrease in SBP post-training (b, $p < 0.05$). However, in the continuous training group a correlation was not observed (c, $p > 0.05$). *d* indicates cohen's effect size.

6.3.3 Cardio-respiratory fitness at pre, mid and post 12-weeks of an interval and continuous exercise training programme in overweight/obese women

Cardio-respiratory fitness was assessed in 17 participants (INT n = 8, CON n = 9) at pre, mid and post-training due to 2 participants at mid-training unavailable for testing and 1 participant's data at post-training excluded due to injury. Absolute $\dot{V}O_{2max}$ significantly increased following training in both groups (p = 0.02) with no time by group interaction (p = 0.17). Absolute $\dot{V}O_{2max}$ was significantly increased from pre to mid-training by 4% (p = 0.01, 95% CI for pre to post-training difference +0.018 to +0.147 $L \cdot min^{-1}$), but no further increases were observed at post-training (Figure 6.8). Relative $\dot{V}O_{2max}$ also increased with training regardless of training group although significance was not reached (p = 0.07, d = 0.4, time by group interaction p = 0.50, Table 6.3). The increase in absolute and relative $\dot{V}O_{2max}$ from pre to post-training was greater in participants with a lower pre-training $\dot{V}O_{2max}$ (absolute $\dot{V}O_{2max}$: r = -0.50 p = 0.04; relative $\dot{V}O_{2max}$: r = -0.46, p = 0.06).

Table 6.3. Cardio-respiratory fitness (mean ± SD) pre, mid and post 12-weeks of either heavy-intensity interval (INT) or continuous (CON) exercise training.

	INT (n = 8)			CON (n = 9)		
	Pre	**Mid**	**Post**	**Pre**	**Mid**	**Post**
Relative $\dot{V}O_{2max}$ ($ml \cdot kg \cdot min^{-1}$)	24.1 ± 2.9	26.0 ± 3.0	25.3 ± 3.3	24.6 ± 2.7	25.0 ± 2.3	25.7 ± 2.3
Lactate threshold (ml)	1077 ± 187	1178 ± 279	1169 ± 247	1181 ± 239	1191 ± 227	1181 ± 183
Lactate threshold (%)	52 ± 7	54 ± 4	55 ± 8	56 ± 6	55 ± 5	54 ± 5

$\dot{V}O_{2max}$ = maximal oxygen uptake

The estimated LT did not change with training in either group (LT (ml) p = 0.35, LT as a percentage of $\dot{V}O_{2max}$ p = 0.99) and there was no time by group interaction (p > 0.05, Table 6.3). The RI test duration and WR_{peak} significantly

increased throughout the training programme by 10% and 11%, respectively (RI duration p = 0.0001, WR_{peak} p = 0.0001) regardless of group (time by group interaction $p > 0.05$). Significant increases were observed between pre to mid-training (RI duration p = 0.01, WR_{peak} p = 0.0001), mid to post-training (RI duration p = 0.03, WR_{peak} p = 0.03) and pre to post-training (RI duration p = 0.001, WR_{peak} p = 0.0001, Figure 6.8).

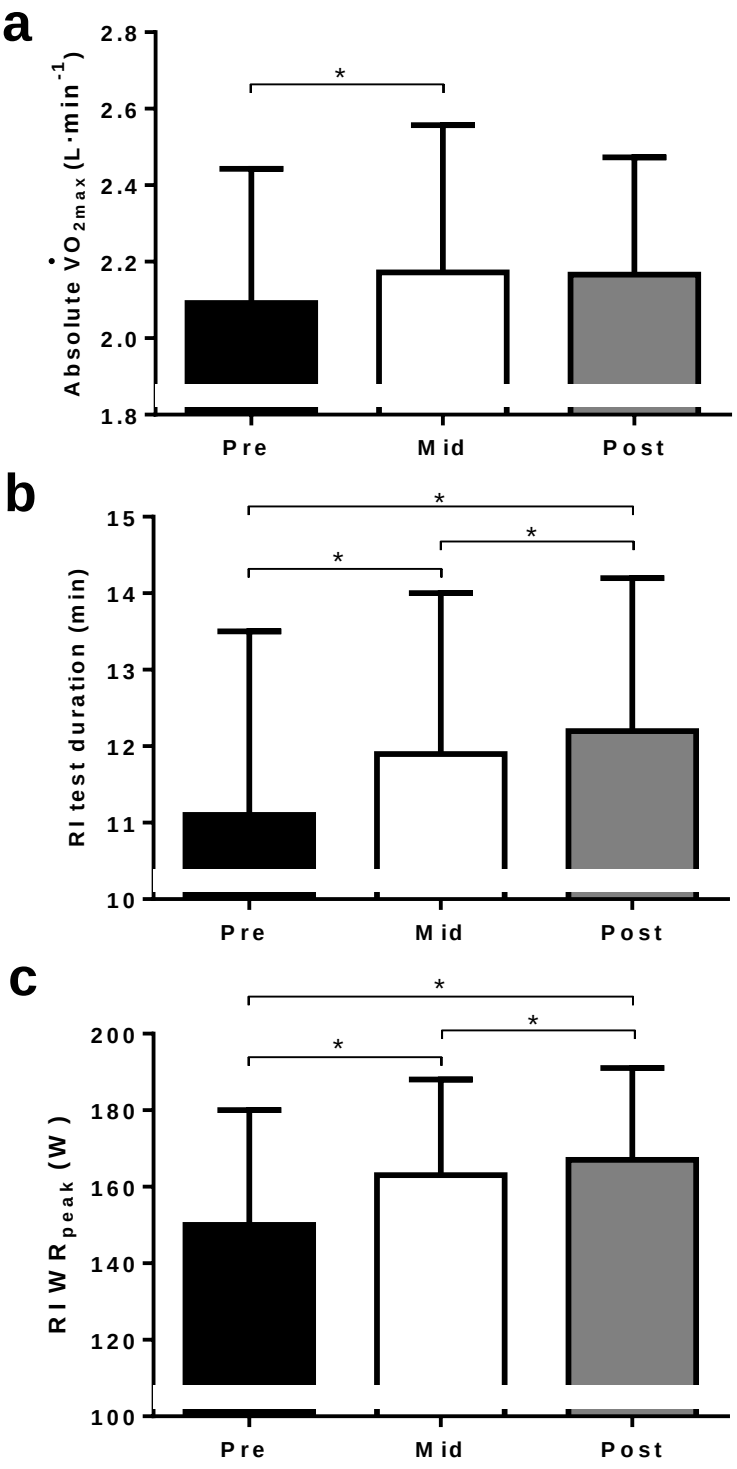

Figure 6.8. Changes in cardio-respiratory fitness at pre, mid and post interval and continuous training. * indicates a significant difference (p < 0.05). a) Absolute maximal oxygen uptake ($\dot{V}O_{2max}$) increased from pre to mid and pre to post-training (p < 0.05). The ramp incremental (RI) test duration (b) and RI work-rate peak ($WR_{peak,}$ c) increased between each time point of the training programme (p < 0.05). There were no group by time interactions (p > 0.05).

6.3.4 The effect of a 12-week interval and continuous exercise training programme on brachial artery endothelial function in overweight/obese women

Resting brachial artery diameter and time from cuff release to peak dilation did not significantly change from pre to post-training in either group ($p > 0.05$, Table 6.4). Relative and absolute FMD showed a close to significant reduction following training ($FMD_{rel:}$ $p = 0.07$, Figure 6.9a; FMD_{abs} $p = 0.05$, Table 6.4). Although, there was no time by group interaction this reduction appeared to be driven by ~2% decrease in the INT training group, whereas there was little change following CON training (Figure 6.9a). Post-hoc paired t-tests revealed a significant decrease in FMD_{rel} in the INT training group ($p = 0.03$, 95% CI for pre to post-training difference -3.6 to -0.3%) but no change following CON training (95% CI for pre to post-training difference -2.5 to +1.8%, $p = 0.73$). Participants with a smaller FMD at pre-training tended to exhibit an increase in FMD following training ($r = -0.49$, $p = 0.03$, Figure 6.9b).

Table 6.4. Brachial artery endothelial function (mean ± SD) pre and post 12-weeks of either heavy-intensity interval (INT) or continuous (CON) exercise training.

	INT (n = 10)		CON (n = 10)	
	Pre	**Post**	**Pre**	**Post**
Resting diameter (mm)	3.40 ± 0.70	3.49 ± 0.63	3.43 ± 0.49	3.44 ± 0.56
Time from cuff release to peak diameter (reciprocal transformed)	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.02
Insonation angle (°)	68 ± 1	68 ± 1	68 ± 1	68 ± 1
Absolute FMD (mm) *	0.28 ± 0.09	0.21 ± 0.07	0.25 ± 0.10	0.24 ± 0.11
VTI_{peak} (cm)	1727 ± 542	1262 ± 405	1499 ± 413	1559 ± 687
Peak reactive hyperaemia ($cm \cdot s^{-1}$)	92 ± 27	83 ± 21	99 ± 19	95 ± 21
Peak shear rate (s^{-1})	2232 ± 717	1950 ± 573	2356 ± 637	2287 ± 735
AUC_{peak} (a.u.)	40698 ± 14395	29709 ± 11103	35921 ± 12231	35636 ± 15245

* indicates a significant main time effect ($p < 0.05$). FMD = flow-mediated dilation, VTI_{peak} = velocity time integral from cuff deflation to peak diameter and AUC_{peak} = shear rate area under the curve from cuff deflation to peak diameter.

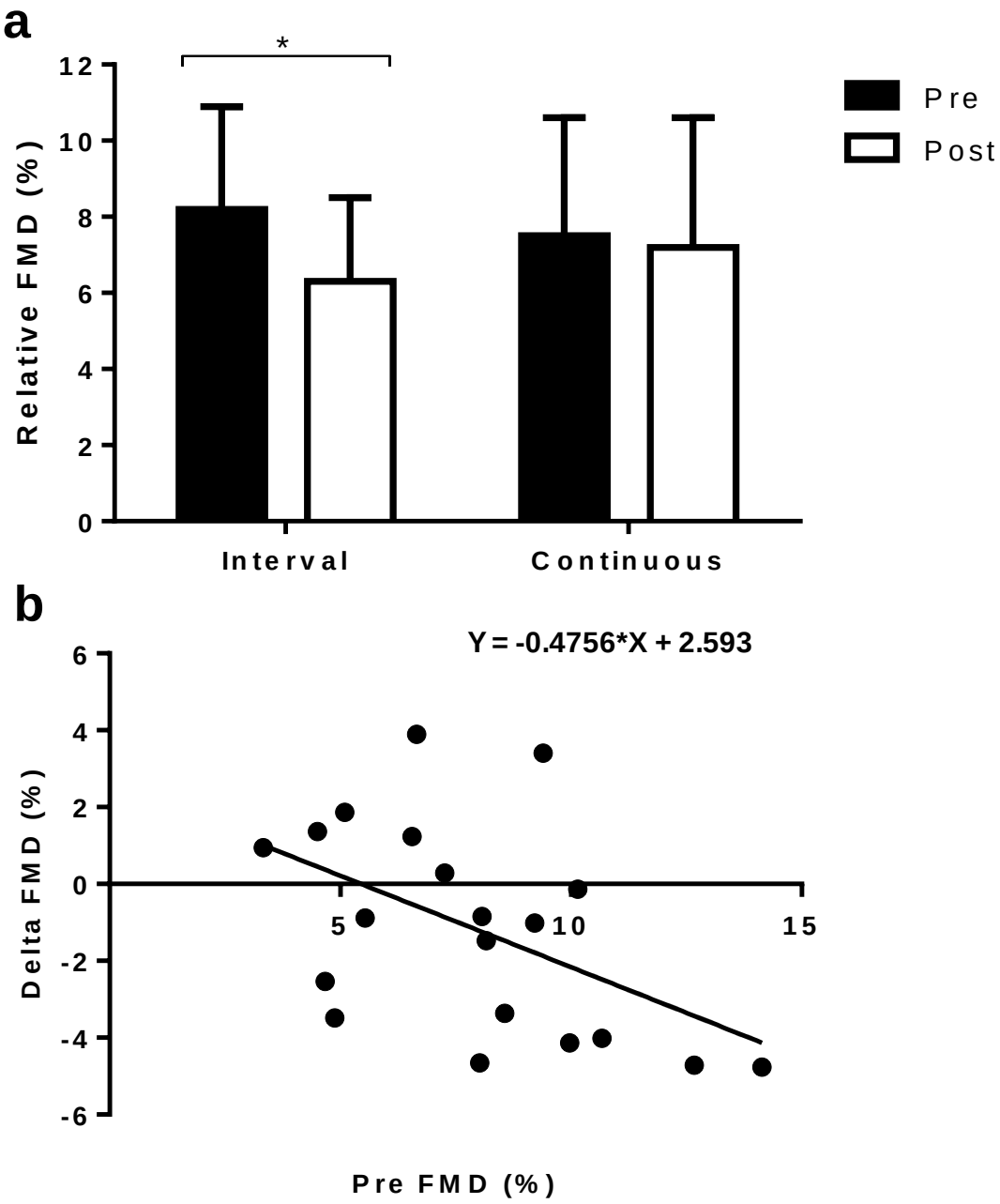

Figure 6.9. Brachial artery flow-mediated dilation (mean ± SD) following interval and continuous exercise training. a) * indicates a significant decrease in FMD from pre to post-training following interval exercise (*p* = 0.03). b) A significant negative correlation was observed between pre-training FMD and the pre to post-training change (r = -0.49, *p* = 0.03).

The VTI_{peak}, AUC_{peak} and peak reactive hyperaemia and shear rate were not significantly changed with training (VTI_{peak} p = 0.20, AUC_{peak} p = 0.15, Peak reactive hyperaemia p = 0.20, peak shear rate p = 0.11) and there was no time by group interaction (p > 0.05, Table 6.4). However, the VTI and shear rate AUC for 60 s showed a close to significant trend for a reduction following

training in both groups (VTI_{60} p = 0.09, 95% CI for pre to post-training difference -440 to +33 cm; AUC_{60} p = 0.05, 95% CI for pre to post-training difference -11128 to +52 a.u.) with no time by group interaction (p > 0.05). However, this decrease was larger following INT training (VTI_{60} p = 0.09, 95% CI for pre to post-training difference -799 to +67 cm; AUC_{60} p = 0.06, 95% CI for pre to post-training difference -20053 to +592 a.u.; Figure 6.10a,c) compared with CON training (VTI_{60} p = 0.73, AUC_{60} p = 0.64). Furthermore, the VTI_{90} showed a close to significant reduction and AUC_{90} significantly reduced following training in both groups (VTI_{90} p = 0.06, 95% CI for pre to post-training difference -537 to +15 cm; AUC_{90} p = 0.04, 95% CI for pre to post-training difference -13858 to -406 a.u.) with a close to significant time by group interaction (VTI_{90} p = 0.09, AUC_{90} p = 0.08, Figure 6.10b,d). Post-hoc paired t-tests revealed a significant reduction in AUC_{90} (p = 0.04, 95% CI for pre to post-training difference -25350 to -704 a.u.) and a close to significant decrease in VTI_{90} (p = 0.05, 95% CI for pre to post-training difference -994 to +7 cm) following INT training but no significant change following CON training (VTI_{90} p = 0.84, AUC_{90} p = 0.72). Absolute FMD showed a significant positive correlation with shear rate AUC at the post-training time point only (AUC_{peak} r = 0.61, p = 0.005; AUC_{60} r = 0.52, p = 0.02; AUC_{90} r = 0.55, p = 0.01). Therefore, FMD was not normalised to shear rate AUC. The change from pre to post-training in FMD did not correlate with the change from pre to post-training in peak reactive hyperaemia, peak shear rate and the VTIs and shear rate AUCs (p > 0.05). Assessment of brachial artery endothelial function was completed between weeks 3-4 of training. However, due to menstrual cycle differences from pre-training and participant unavailability, this assessment was not completed in 5 participants (INT n = 6, CON n = 9). Therefore a separate ANOVA was conducted in 15 participants to

compare differences in endothelial function at pre, mid and post-testing. There were no changes in any variables between the time points and no time by group interactions ($p > 0.05$).

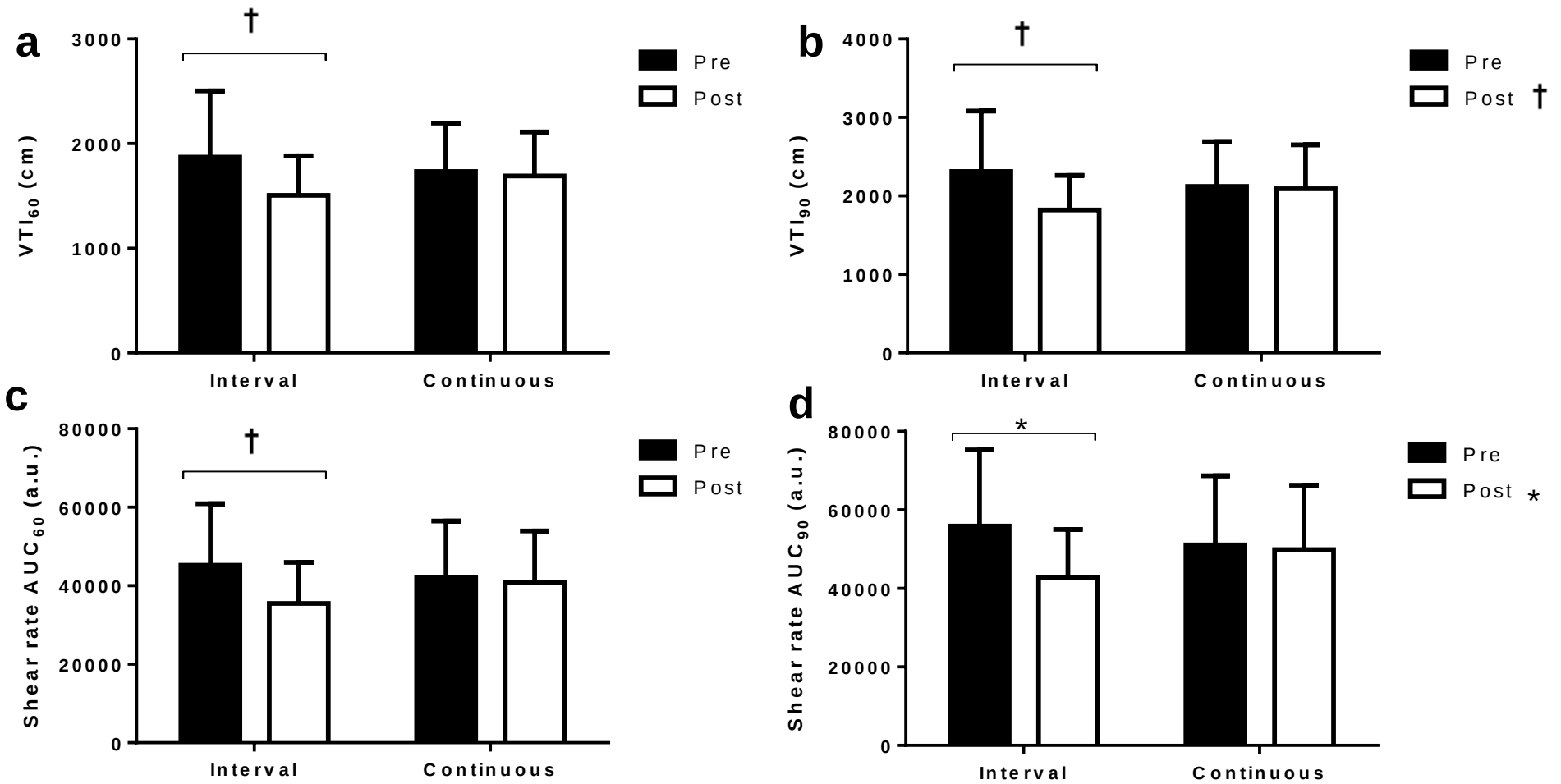

Figure 6.10. The velocity time integrals (VTI_{60} and VTI_{90}) and shear rate area under the curves (AUC_{60} and AUC_{90}) following interval and continuous training. * indicates a significant difference ($p < 0.05$). † indicates a close to significant difference ($p \leq 0.09$). VTI_{60} (a) and AUC_{60} (c) reduced following interval training ($p > 0.05$). VTI_{90} (b) reduced following training with a greater decrease following interval training ($p = 0.05$) than continuous training ($p > 0.05$). AUC_{90} (d) significantly reduced following training ($p = 0.04$) with a greater reduction following interval training ($p = 0.04$, group by time interaction $p = 0.08$).

6.3.5 The effect of a 12-week interval and continuous exercise training programme on arterial stiffness in overweight/obese women

Due to difficulties with tonometry recordings, one participant was excluded from PWV_{cr} analysis in the CON group and two participants were excluded from PWV_{bf} analysis in the CON group. Peripheral and central PWV did not change following either INT or CON exercise training (PWV_{cr} p = 0.13, PWV_{bf} p = 0.60) with no time by group interaction (p > 0.05, Table 6.5). Carotid arterial stiffness did not change following training in either group (IMT p = 0.50, carotid artery SBP p = 0.11, Carotid artery PP p = 0.25, ΔCSA p = 0.64, CSC p = 1.00, distensibility p =0.49, SI p = 0.95, Table 6.5) with no time by group interactions (p > 0.05).

Table 6.5. Arterial stiffness (mean ± SD) pre and post either interval (INT) or continuous exercise training (CON).

	INT (n = 10)		CON (n = 10)[1]	
	Pre	**Post**	**Pre**	**Post**
PWV_{cr} ($m \cdot s^{-1}$) CON n = 9	6.9 ± 0.9	7.2 ± 1.0	7.5 ± 0.7	7.8 ± 1.0
PWV_{bf} ($m \cdot s^{-1}$) CON n = 8	7.6 ± 1.2	8.0 ± 0.8	8.7 ± 2.2	7.8 ± 1.1
Carotid artery IMT (mm)	0.39 ± 0.04	0.41 ± 0.04	0.44 ± 0.05	0.44 ± 0.05
Carotid artery SBP (mmHg)	112 ± 11	108 ± 8	113 ± 13	111 ± 14
Carotid artery PP (mmHg)	31 ± 4	29 ± 5	31 ± 8	30 ± 6
Carotid ΔCSA within heart cycle (mm^2)	4.3 ± 0.6	4.4 ± 0.9	4.7 ± 1.4	4.5 ± 1.1
Carotid artery CSC (mm^2/mmHg)	0.14 ± 0.03	0.16 ± 0.04	0.17 ± 0.08	0.15 ± 0.04
Carotid artery DD (mm/mmHg)	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.003	0.004 ± 0.001
Carotid artery SI (a.u.)	5.2 ± 1.2	5.3 ± 1.2	5.5 ± 2.3	5.5 ± 1.0

[1] indicates that 10 participants were included in the analysis from the CON group except for PWV. PWV_{cr} = carotid-radial pulse wave velocity, PWV_{bf} = brachial-foot pulse wave velocity, IMT = intima-media thickness, SBP = systolic blood pressure, PP = pulse pressure, ΔCSA = difference between maximum and minimum cross-sectional area, CSC = cross-sectional compliance, DD = distensibilty, SI = β-stiffness index.

6.3.6 The effect of a 12-week interval and continuous exercise training programme on cytokine concentrations and CAC number

CAC number was analysed from 16 participants (INT n = 6, CON n = 10) as pilot work for the enumeration protocol was being undertaken and 2 participants did not want to give blood. Following training, $CD34^+$ and $CD34^+CD45^{dim}KDR^+$ CACs did not change ($CD34^+$ p = 0.17, $CD34^+CD45^{dim}KDR^+$ p = 0.25, Table 6.6) in either group (time by group interaction $p > 0.05$). Results from the ANCOVA show a significant increase in $CD34^+CD45^{dim}$ CACs following training (p = 0.03). Although a time by group interaction was not observed (p = 0.50), there was a large effect size for an increase following INT (d = 1.0) but no change following CON (Figure 6.11a). Post-training SD was used in the effect size calculation as the pre-training values did not have a SD due to covariate analysis. The pre-training values had a significant impact on the effect of the training on $CD34^+CD45^{dim}$ CACs (time by pre-training $CD34^+CD45^{dim}$ CACs interaction p = 0.04).

Table 6.6. Circulating angiogenic cell (CAC) number, plasma SDF-1α and leukocyte number (mean ± SD) pre and post either interval (INT) or continuous exercise training (CON).

	INT[1]		CON (n = 10)	
	Pre	**Post**	**Pre**	**Post**
$CD34^+$ CACs (/ 10^5 leukocytes) INT: n = 6	2.9 ± 0.2	3.2 ± 0.6	3.2 ± 0.4	3.2 ± 0.4
$CD34^+CD45^{dim}KDR^+$ CACs (/ 10^5 leukocytes) INT: n = 6	0.4 ± 0.3	0.5 ± 0.4	0.5 ± 0.3	0.7 ± 0.6
Plasma SDF-1α (pg/ml) INT: n = 8	1436 ± 174	1399 ± 238	1440 ± 315	1619 ± 222
Leukocytes (/ml of blood) INT: n = 7	6,078571 ± 2,292534	6,253837 ± 1,094102	5,874286 ± 1,259923	5,570000 ± 991408

[1] indicates that the number of participants included in the analysis from the INT group are specified for each variable individually. SDF-1α = stromal derived factor-1 alpha.

Plasma VEGF and SDF-1α were analysed from 17 and 18 participants respectively, due to reasons previously stated. Following exercise training plasma VEGF increased significantly in both groups (p = 0.02, group by time interaction p = 0.87; Figure 6.11b), whereas no changes were observed in plasma SDF-1α (p > 0.05, Table 6.6). Changes in CAC number following training were not related to changes in FMD or plasma VEGF (p > 0.05). Leukocyte number did not change after either training type (p > 0.05, n =17, Table 6.6).

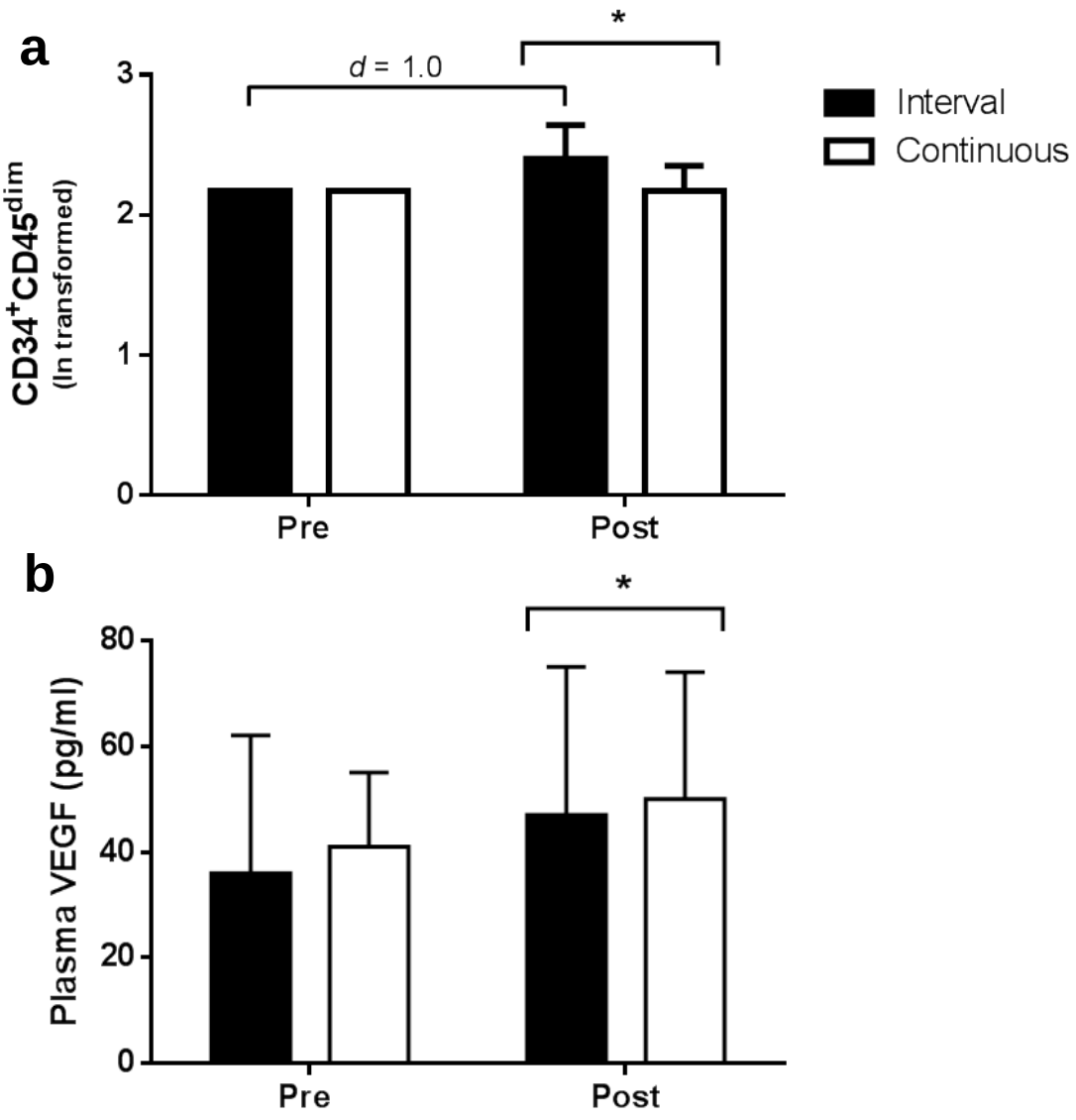

Figure 6.11. CD34+CD45dim cells (a) and plasma VEGF (b, mean ± SD) following interval and continuous exercise training. * indicates a significant difference from pre-training in both groups (p > 0.05). *d* indicates cohen's effect size.

6.3.7 The effect of a 12-week interval and continuous exercise training programme on CAC culture growth characteristics

During post-testing the cell culture incubator became infected. Therefore, many participants had to be excluded from analysis due to unreliable data or cell death. Data analysed from 11 participants showed that the number of adherent live cells at the end of cell culture on day 7 was not significantly different between pre and post training in either training group (p = 0.59, time by group interaction p = 0.62, Table 6.7). The number of non-adherent cells collected during culture was analysed in 14 participants on day 2 only, due to the reasons previously stated. The total number of non-adherent cells and the percentage of dead non-adherent cells also did not change with training in either group (p > 0.05, Table 6.7).

Table 6.7. Cell culture growth characteristics (mean ± SD) pre and post either interval (INT) or continuous exercise training (CON).

	INT		CON	
	Pre	**Post**	**Pre**	**Post**
Day 7 adherent cells / well (INT: n = 6; CON: n = 5)	130000 ± 23476	131111 ± 79740	119400 ± 43536	145000 ± 41046
Day 2 total non-adherent cells / well (INT: n = 5; CON: n = 9)	1,946667 ± 787002	1,636000 ± 653853	2,089259 ± 414580	1,832037 ± 767422
Day 2 percentage of dead non-adherent cells / well (INT: n = 5; CON: n = 9)	8.6 ± 5.4	11.3 ± 3.7	7.3 ± 3.9	8.6 ± 6.2

Cell area was measured on day 2 (n = 14), 4 (n = 11) and 7 (n = 11) of culture. There was no difference between pre and post-training on day 2 (p = 0.40), day 4 (p = 0.53) and day 7 (p = 0.25) in either training group (time by group interaction p > 0.05). The exercise training group average percentage change in cell area from pre to post-training was not significantly different between training groups during cell culture (p > 0.05) as illustrated in Figure 6.12.

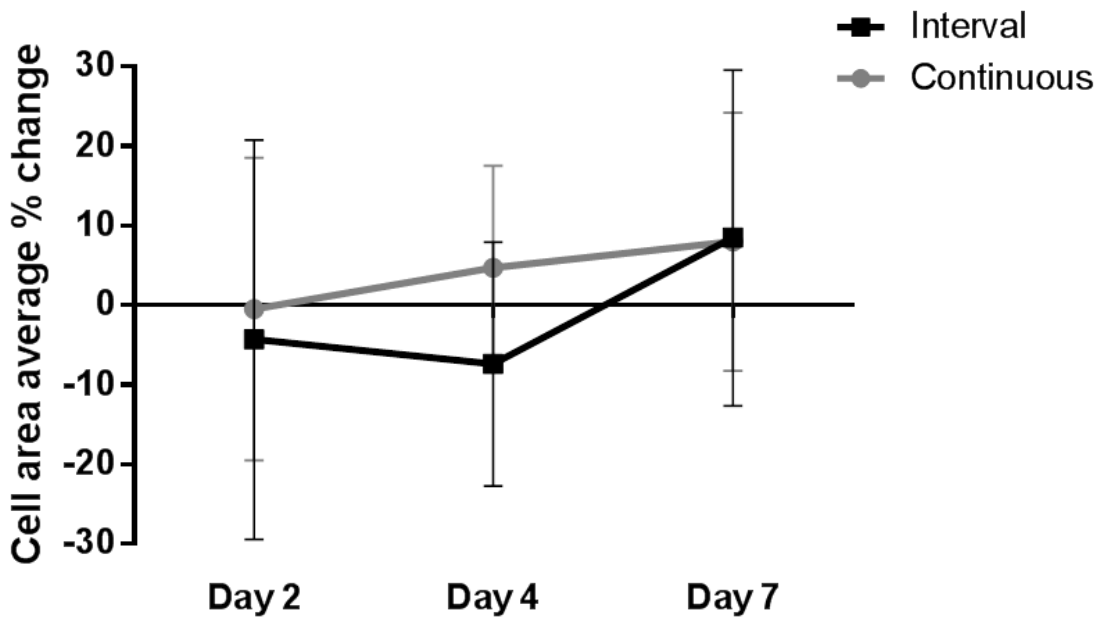

Figure 6.12. The group average (± SD) percentage change in cell area from pre to post-training during cell culture in the interval (n = 6) and continuous (n = 8) training groups. There was no difference between the training groups on day 2 (*p* = 0.75), day 4 (*p* = 0.19) and day 7 (*p* = 0.97) on cell culture.

6.3.8 The effect of a 12-week interval and continuous exercise training programme on CAC paracrine activity and adhesive functional ability

Due to the cell culture incubator infection, undetectable values in the ELISA kit analysis of cytokines and two participants unwilling to give blood, CAC secretion of VEGF, SDF-1α and G-CSF was analysed from 11, 14 and 8 participants, respectively. Following training in both groups there was no significant change in secretion of VEGF (*p* = 0.29), SDF-1α (*p* = 0.53) and G-CSF (*p* = 0.61, Table 6.8). A close to significant time by group interaction was observed for G-CSF (*p* = 0.06). However, the post-training changes were very small (1-2 pg/10^5 cells) in both groups (95% CI for difference INT: -7 to +2 pg/10^5 cells; CON: -0.1 to +3 pg/10^5 cells).

Table 6.8. Cytokine secretion from cell culture conditioned medium (mean ± SD) pre and post either interval (INT) or continuous exercise training (CON).

	INT		CON	
	Pre	**Post**	**Pre**	**Post**
Secreted VEGF (pg/10^5 cells) INT: n = 5; CON: n = 8	11 ± 7	6 ± 2	8 ± 6	9 ± 7
Secreted SDF-1α (pg/10^5 cells) INT: n = 5; CON: n = 9	552 ± 210	589 ± 415	640 ± 309	739 ± 482
Secreted GCSF (pg/10^5 cells) INT: n = 4; CON: n = 4	4 ± 3	2 ± 1	3 ± 2	4 ± 3

VEGF = vascular endothelial growth factor, SDF-1α = stromal derived factor- 1 alpha and GCSF = granulocyte colony stimulating factor.

The ability of cultured CACs to adhere to a monolayer of saphenous vein VSMCs was analysed from 8 participants (INT n = 4, CON n = 4) due to the reasons previously stated. At the 20 min time point, CAC adhesion at pre-training was significantly higher in the CON group compared to the INT group ($p < 0.05$). Consequently, the pre-training values were added as a covariate to the ANOVA for the 20 min time point only. A greater number of CACs adhered to VSMCs following INT training at the 10 min (time by group interaction $p = 0.04$) and 30 min (time by group interaction $p = 0.08$) time points only. As illustrated in Figure 6.13 the average percentage change in CAC adhesion was significantly higher following INT training at the 10 min time point ($p = 0.03$) whereas there was little change with CON training. There were no significant differences between the training groups at any other time point ($p > 0.05$).

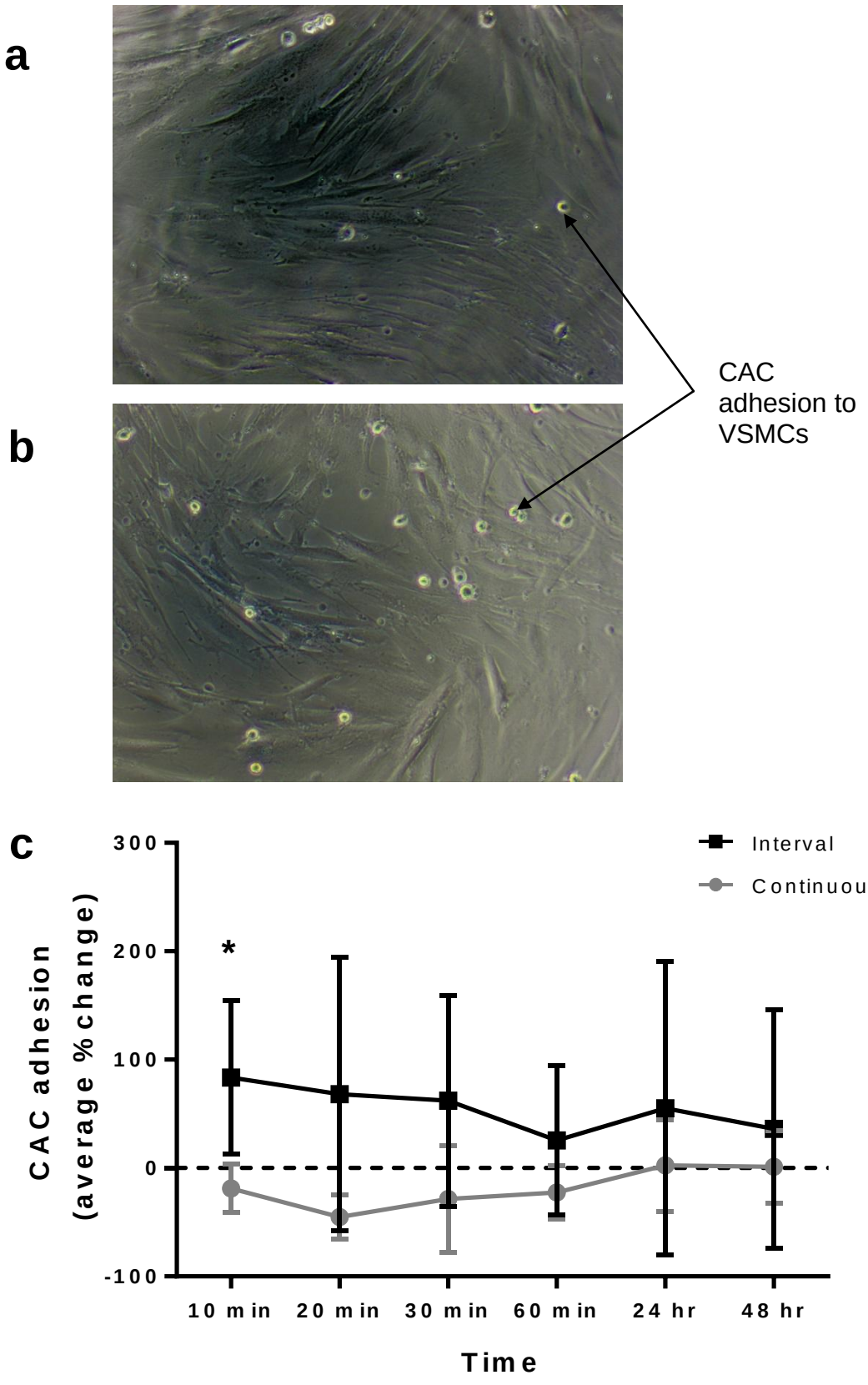

Figure 6.13. CAC adhesion to a monolayer of VSMCs. Images of the adherence of CACs to VSMCs at pre (a) and post (b) training from one participant in the interval training group. c) A time course of the group average (± SD) percentage change in CAC adherence to VSMCs from pre to post interval (n = 4) and continuous (n = 4) training. * indicates a significant difference between the training groups ($p < 0.05$).

6.4 Discussion

The present study was the first to match interval and continuous exercise training for the heavy-intensity domain and compare their effects on vascular health in overweight/obese women. The main findings were that heavy-intensity interval exercise training had superior effects on CAC number and function but conversely reduced brachial artery FMD, whereas intensity and work matched continuous exercise training had no effect. Nonetheless, continuous exercise training improved cardio-respiratory fitness and plasma VEGF to the same extent as interval exercise, suggesting that increases in these parameters were unrelated to the mechanisms responsible for altering FMD and CAC number and function.

6.4.1 Interval exercise training reduced brachial artery FMD whereas continuous exercise training had no effect

Following 12 weeks of heavy-intensity cycling training in middle-aged overweight/obese women, interval exercise reduced upper limb brachial artery FMD whereas continuous exercise had no effect. Given that improvements in brachial artery endothelial function following exercise training are mediated by increased nitric oxide bioavailability induced by increases in shear stress during the exercise sessions (Tinken *et al.*, 2010; Birk *et al.*, 2012); the absence of improvements following continuous exercise training might be related to an insufficient volume of shear stress experienced by participants during the exercise sessions. Another potential explanation might be related to the time course for upper limb arterial adaptations to lower limb exercise. Arterial functional alterations occur rapidly with exercise training followed by structural adaptations. This was evidenced in humans where brachial artery FMD and maximal dilator capacity, as a measure of endothelial function and arterial

structural changes respectively, were assessed every 2 weeks during an 8 week cycling and running training programme in healthy males (Tinken *et al.*, 2008). As shown in Figure 6.14, FMD increased after 2 weeks of training and subsequently declined at 8 weeks to the same levels as the pre-training values. In contrast, maximal dilator capacity continued to increase throughout the training programme, thereby suggesting that endothelial function was reducing to pre-training levels due to arterial remodelling. Hence, this might explain why no changes in endothelial function were observed following 12 weeks of continuous exercise training. FMD was measured at 4 weeks and no changes were found, however, at this point the artery might have remodelled, therefore negating improvements in function. However, this does not explain why brachial artery FMD was reduced following work and intensity matched interval exercise training.

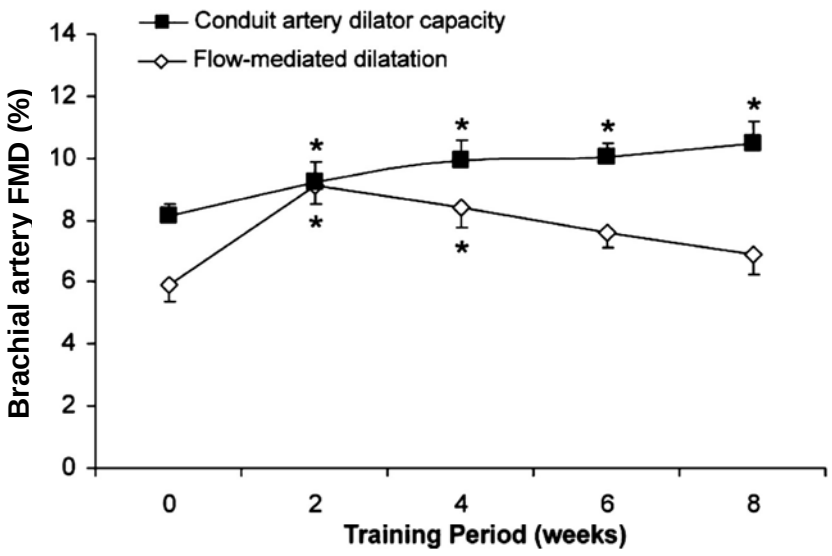

Figure 6.14. Brachial artery flow-mediated dilation (FMD) and maximal dilator capacity (mean ± standard error mean) across an 8-week cycling training programme. * indicates a significant difference from pre-training (*p* < 0.05). Reproduced from Tinken *et al*., (2008).

The decrease in brachial artery FMD following interval exercise training was surprising as previous studies have reported improvements following 12 weeks

of interval exercise training in obese adults (Schjerve *et al.*, 2008; Tjønna *et al.*, 2008; Stensvold *et al.*, 2010) and after 8 weeks in obese rats (Haram *et al.*, 2008). However, the previous studies in humans used upper arm inflation to create ischaemia during the FMD assessment, which is not totally endothelial and nitric oxide dependent, (Doshi *et al.*, 2001). Thus, the increase in FMD post-interval training in the previous studies (Schjerve *et al.*, 2008; Tjønna *et al.*, 2008; Stensvold *et al.*, 2010) may not have reflected increased endothelial function. Moreover, a different type of interval exercise was adopted in the previous studies, which involved 4 x 4 min periods at ~90% HR_{max} separated by 3 min recovery at ~70% $HR_{max.}$ In the present study, interval exercise sessions involved repeated duty cycles of 40 s at 70%Δ work-rate followed by 80 s active recovery at 20 W. Thus, the differences in intensity and work-rate profiles during interval exercise between the present and previous studies might have contributed to the different results observed.

A reduction in brachial artery endothelial function has been reported following aerobic exercise training in one previous study (Bergholm *et al.*, 1999). In this previous study, the increase in blood flow in response to acetylcholine infusion in the forearm, decreased following 3 months of continuous running exercise, involving four 1 hr sessions per week at 70-80% $\dot{V}O_{2max}$ in healthy males (Bergholm *et al.*, 1999). The impairment in endothelial function was explained by an increase in oxidative stress and a reduction in anti-oxidant enzymes post-training. This was also observed in another study in which oxidative stress increased and acetylcholine-induced vasodilation remained unchanged, following high-intensity continuous exercise training [12 weeks of 30 min cycling at 75% $\dot{V}O_{2max}$ on 5-7 days/week; Goto *et al*., (2003)]. However, an increase in oxidative stress following interval exercise training in the present study is

unlikely, given that 1) interval and continuous exercise sessions were matched for intensity and work and FMD did not change with continuous exercise training, and 2) CAC number and function appeared to improve following interval exercise training, with reduced oxidative stress a suggested mechanism (Fernandes *et al.*, 2012). Therefore, other explanations could be related to changes in vessel vasomotor tone.

Following interval exercise training a reduction in post-ischaemic shear rate AUC_{60+90} was observed in combination with the decrease in FMD. Given that shear stress is the stimulus for FMD, the reduced shear rate AUC may in part explain the decreased FMD. Increased forearm vascular resistance can reduce brachial artery shear rate (Simmons *et al.*, 2011). However, augmented peripheral resistance post-exercise training is unlikely as brachial artery SBP showed a trend for a reduction following interval exercise, which is usually related to reduced peripheral resistance and/or increased vessel function (Guimaraes *et al.*, 2010). Forearm vascular remodelling could potentially explain the reduction in shear rate AUC. If participants in the interval exercise group had lost lean or fat mass from the forearm, it could be postulated that the ischaemic stimulus would be smaller post exercise and thus, the resultant shear rate and FMD would also be reduced. However, further studies would be required to validate this theory.

Another potential explanation for the reduction in FMD post-interval exercise training, could be related to enhanced brachial artery vasomotor control. In a previous study we observed no changes in FMD but reported a greater constriction to reduced flow during the period of forearm occlusion in the brachial artery following interval exercise of the same nature as the present study [i.e. 1:2 work:recovery duty cycles (Rakobowchuk *et al.*, 2012)]. As low-

flow mediated constriction was not measured in the present study we cannot identify whether vasoconstrictor tone and endothelial sensitivity was enhanced with exercise training. Furthermore, maximal dilator capacity, which indicates arterial structural changes was not measured in the present study. If the maximal dilator capacity had increased, then the reduction in FMD following interval exercise training would not indicate a reduction in endothelial function, but instead reflect arterial remodelling. The fluctuations in blood flow associated with variable work-rates during interval exercise sessions as mentioned in previous chapters, may explain the differences observed between interval and continuous training. However, further studies are required to measure blood flow during this type of interval exercise, and to examine the effects of these profiles on endothelial cells *in vitro*.

6.4.2 Increased cardio-respiratory fitness following both interval and continuous exercise training

Both heavy-intensity interval and continuous exercise training increased $\dot{V}O_{2max}$ and indicators of exercise tolerance (RI test duration and WR_{peak}), which is consistent with previous interval and continuous exercise training studies in obese adults (Schjerve *et al.*, 2008; Tjønna *et al.*, 2008). Given that both training groups were in the heavy-intensity domain, the intensity of the exercise was likely the mechanism for the adaptation and not the interval or continuous nature of the exercise. Further evidence in support of intensity as the mediator for enhanced cardio-respiratory fitness is provided by a study which reported an increase in $\dot{V}O_{2max}$ following heavy-intensity interval exercise, but no change following moderate-intensity interval exercise involving the same 1:2 ratio of work:recovery duty cycles (Rakobowchuk *et al.*, 2012). Mechanisms underlying the improvement in cardio-respiratory fitness involve an increased delivery of

blood in combination with an increased utilisation of oxygen to working muscles. In obese adults following exercise training, an increase in stroke volume and skeletal muscle mitochondrial capacity has been reported (Tjønna *et al.*, 2008). Other mechanisms relate to an increased endothelial vasodilation and reduced arterial stiffness of the lower limb arteries, which would potentially enable a greater delivery of oxygen to the periphery, as reported in the popliteal artery following cycling exercise training (Rakobowchuk et al., 2008). The increase in $\dot{V}O_{2max}$ occurred within the first 6 weeks of exercise training with no further improvements between mid and post-training. Although the increase in $\dot{V}O_{2max}$ was small in the present study, it is reported that an increase in $\dot{V}O_{2max}$ by 1 ml·kg·min^{-1} reduces the risk of all-cause and cardiovascular related death by 15% (Keteyian *et al.*, 2008). Thus, the exercise training adopted in the present study might be prescribed to women with low $\dot{V}O_{2max}$ values and poor cardiovascular health.

6.4.3 Unchanged arterial stiffness following interval and continuous exercise training

No changes in central and peripheral PWV or carotid arterial stiffness were observed following either heavy-intensity interval and continuous exercise training in overweight/obese women. Improvements in arterial stiffness are determined by functional and structural alterations in the vessel wall. The increase in pulsatile pressure on the arterial wall during exercise is suggested to initiate increased elastin synthesis by vascular smooth muscle cells (Matsuda *et al.*, 1993), and/or decreased cross-linking of connective tissue (Joyner, 2000), thus reducing arterial stiffness. It could be suggested that in the present study the intra-luminal pressure during exercise was not great enough to stimulate these structural adaptations, or that a greater volume of exercise is required in

overweight/obese women for arterial stiffness alterations to occur. However, reductions in arterial stiffness have been observed in obese adults following 12 weeks of continuous running exercise (Miyaki *et al.*, 2009b). The increase in carotid arterial cross-sectional compliance and reduction in β-stiffness index were concomitant with an increase in plasma nitric oxide and reduction in endothelin-1. The alteration in systemic redox status was suggested to enhance arterial vasomotor tone, contributing to the reduced arterial stiffness. Therefore, the lack of improvement in endothelial function in the present study might have impaired reductions in arterial stiffness. Alternatively, the contrasting results in the present study might be explained by no changes to BMI, as the previous study observed reduced BMI, fat mass and LDL and total cholesterol levels, which will likely have contributed to an increased arterial function by reducing inflammation (Miyaki *et al.*, 2009b). A greater exercise volume in the previous study (40-60 min, 3 days/week) compared to the current study most likely explains the weight loss experienced by participants in the previous study. Thus, for improvements in arterial stiffness following exercise training in overweight/obese women, a reduction in BMI and fat mass might be required, which could be achieved by a greater exercise training volume/stimulus.

6.4.4 Interval exercise training but not continuous increased circulating angiogenic cell number

An increased number of CACs following 12 weeks of continuous exercise training has been reported in coronary artery disease (Steiner *et al.*, 2005), chronic heart failure (Erbs *et al.*, 2010) and obese patients (Cesari *et al.*, 2012). However, the present study was the first to compare the effects of 12 weeks of interval and continuous exercise training on CAC number in a population of overweight/obese women. Despite only small changes, interval exercise training

appeared to increase $CD34^{+}CD45^{dim}$ cell number whereas continuous exercise training had no effect. There were no changes in $CD34^{+}CD45^{dim}KDR^{+}$ cells. Although the increase in CACs was not necessarily endothelial specific, $CD34^{+}$ cells are haematopoietic stem cells which have been shown to migrate and adhere to capillaries in an ischaemic hind-limb mouse model (Asahara *et al.*, 1997) and are negatively correlated with CVD risk factors (Fadini *et al.*, 2006b). Thus, the increase following interval exercise training may partly ameliorate CVD risk factors by contributing to vascular repair or angiogenesis. However, as only a small increase was noted, the impact on vascular health is unclear. Given that larger changes in CAC number were observed in participants with smaller pre-training numbers, the magnitude of the increase may be larger or have a greater impact in obese women with poorer cardiovascular health. Moreover, larger increases might be gained if exercise training was combined with weight loss, as greater reductions in BMI correlated with larger increases in CD34+ cells following a 6 month weight loss diet in obese adults (Muller-Ehmsen *et al.*, 2008). Nevertheless, in a population of healthy obese women, the increased CACs following interval exercise training indicates a greater capacity to maintain vascular homeostasis if required. Future studies investigating exercise training and CAC number should focus on whether increases have a beneficial impact on vascular health and repair. For example by isolating CACs pre and post-training and infusing the cells to ischaemic hind-limb rodent models or to artificially injured vessels.

The mechanisms responsible for exercise training induced increases in CAC number include an increase in nitric oxide bioavailability (Steiner *et al.*, 2005) through an eNOS dependent pathway (Laufs *et al.*, 2004) and/or a reduction in inflammation and oxidative stress (Erbs *et al.*, 2010; Fernandes *et al.*, 2012).

This altered anti-atherogenic environment may contribute to enhanced CAC survival through an increase in telomerase activity (Werner *et al.*, 2009) and reduced apoptosis (Laufs *et al.*, 2004). Although endothelial-dependent vasodilation in the brachial artery which reflects nitric oxide bioavailability did not increase following interval exercise training, this might not reflect systemic nitric oxide bioavailability, as vasomotor tone can differ from limb to limb (Thijssen *et al.*, 2011b). Therefore, it could be suggested that interval exercise increased nitric oxide bioavailability and reduced oxidative stress systemically, contributing to an increased mobilisation of CACs.

The increase in CACs following interval exercise cannot be explained by the intensity of the exercise given that intensity and work matched continuous exercise had no effect on CAC number. Additionally, differences in the haemodynamics between interval and continuous exercise does not explain the contrasting results, as moderate-intensity continuous exercise training increased CAC number in overweight/obese adults in a previous study (Cesari *et al.*, 2012). An alternative explanation could be the duration of the exercise sessions. Following an acute bout of moderate-intensity running, CACs were mobilised following a 30 min bout, but not after a 10 min bout, suggesting that a threshold must be attained for CAC mobilisation (Laufs *et al.*, 2005). Given that in the present study continuous exercise sessions ranged from a minimum 12 min in the first week to a maximum of 30 min in the final week, CACs may not have been acutely mobilised following a continuous exercise session, thus explaining why no changes were observed post-training. However, conversely plasma VEGF which is involved in CAC mobilisation and angiogenesis (Asahara *et al.*, 1999; Zentilin *et al.*, 2006) increased similarly following both interval and continuous exercise training. The relationship between VEGF and

CAC mobilisation is complex as many studies show an exercise-mediated increase in VEGF simultaneously with an increase in CACs (Laufs *et al.*, 2004; Sandri *et al.*, 2005; Sarto *et al.*, 2007; Erbs *et al.*, 2010), whereas others did not (Laufs *et al.*, 2005; Steiner *et al.*, 2005). In the present study, the increase in VEGF was relatively small (~10 pg/ml) in comparison to other studies [~56 pg/ml (Sandri *et al.*, 2005)] and did not correlate with changes in CAC number. Similar discrepancies have been reported elsewhere. In a study investigating capillary angiogenesis in response to differing severities of muscle overload, VEGF was increased to a similar extent in all conditions despite capillary density increasing in only the stronger muscle overload conditions (Egginton *et al.*, 2011). The authors postulated that VEGF creates a pro-angiogenic environment but that other mediating factors such as the balance between pro and anti-angiogenic molecules are required to elicit angiogenesis. Thus, interval exercise potentially due to a longer training session stimulus, might have created a greater pro-angiogenic milieu enabling enhanced CAC mobilisation and survival. Further evidence is provided by an increase in CAC adhesion following interval exercise training.

6.4.5 Interval exercise training but not continuous increased circulating angiogenic cell adhesive ability

CACs contribute to vascular repair and angiogenesis by homing and adhering to the endothelium (Urbich & Dimmeler, 2004) and secreting angiogenic growth factors (Rehman *et al.*, 2003). Although participant numbers were very small, the present study observed an increase in cultivated CAC adhesion to VSMCs at the 10 min time point following interval exercise training but not continuous, without alterations to CAC paracrine activity. The monolayer of VSMCs reflected a denuded vessel lining *in vivo,* as a representation of arterial damage.

Given that cell culture characteristics and cell area did not change post exercise training, any differences between cell culture conditions between pre and post-training cannot explain the increased adhesion observed. Thus, in a population of middle-aged overweight/obese women, interval exercise training potentially increased the capacity for endothelial repair or angiogenesis by improving the ability of CACs to rapidly respond and adhere to damage, injury or other vascular stresses. This is consistent with a previous study following exercise training in adults with the metabolic syndrome, where injection of cultivated CACs into mice with induced carotid arterial injury, improved re-endothelialisation greater than the control group (Sonnenschein *et al.*, 2011). Similar improvements in CAC function following exercise training have been reported in other “at risk” populations. An increased *in vitro* CAC migration and incorporation into tubule structures has been reported following exercise training in peripheral artery disease (Sandri *et al.*, 2005), chronic heart failure (Erbs *et al.*, 2010) and middle-aged and older sedentary males (Hoetzer *et al.*, 2007). The mechanisms responsible for CAC adhesion to the vasculature involve the binding of integrins and receptors present on CACs to ligands expressed in the arterial wall (Urbich & Dimmeler, 2004). Inhibition of SDF-1α receptors CXCR4 and CXCR7 impairs adhesion to collagen and fibronectin *in vitro* (Dai *et al.*, 2011). Additionally, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ blockade reduced carotid artery reendothelialisation in rats (Walter *et al.*, 2002). Indeed, exercise training increased CXCR4 expression on CACs in CAD patients (Sandri *et al.*, 2005). Other reported contributing factors to improved CAC function following exercise training are reduced superoxide production and increased nitric oxide from CACs (Sonnenschein *et al.*, 2011), reduced systemic oxidative stress (Erbs *et al.*, 2010) and increased cell survival (Werner *et al.*, 2009). Thus, interval

exercise might have increased expression of integrins and receptors on CACs by reduced superoxide interaction, enabling rapid adhesion after culture. This might translate into a faster *in vivo* response mechanism to vascular repair or maintenance post exercise training.

The increase in CAC adhesion post interval exercise training but not continuous exercise training is unusual as all studies reporting improvements in CAC function have involved continuous exercise training (Sandri *et al.*, 2005; Hoetzer *et al.*, 2007; Sarto *et al.*, 2007; Erbs *et al.*, 2010; Sonnenschein *et al.*, 2011). However, as intensity was controlled for, the difference in session duration as explained in the previous chapter might explain the divergent results following interval and continuous exercise training.

6.4.6 Conclusions

In conclusion, brachial artery FMD and post-ischaemic shear rate reduced but CAC number and function increased following interval exercise training only. Similar increases in $\dot{V}O_{2max}$ and VEGF were observed following both interval and continuous exercise training, suggesting that the intensity of the exercise determined these adaptations. The differences in session duration and/or the blood flow profile between interval and continuous exercise training may explain the divergent results observed. The reduction in brachial artery FMD does not necessarily indicate poorer vascular health. Brachial artery dysfunction is related to coronary artery dysfunction (Anderson *et al.*, 1995; Takase *et al.*, 1998) but conversely, a recent study indicated that brachial artery FMD might not reflect systemic endothelial function as FMD differs from limb to limb (Thijssen *et al.*, 2011b). Hence, although FMD reduced following interval exercise training in the brachial artery, it may be enhanced elsewhere in the arterial tree, likely in the lower-limbs. Future studies are required to assess

blood flow responses to interval exercise in different arteries and assess endothelial function throughout the arterial tree. Although changes were observed following exercise training, overweight/obese women might require a higher frequency of exercise sessions per week to induce a greater response.

6.4.7 Limitations and future work

The study was limited by the small number of participants that could be included in the cell function analysis due to cell culture contamination. Similarly, cell number was only analysed in six participants in the interval exercise training group. Therefore, strong conclusions cannot be made. Nevertheless, suggestions and hypotheses can be postulated, which require validation through future investigations. The paracrine activity of cells was assessed from the secretion of VEGF, SDF-1α and G-CSF. These cytokines have previously been shown to be secreted into conditioned medium from early culture CACs and are involved in CAC mobilisation, homing and adherence (Rehman *et al.*, 2003; Ceradini *et al.*, 2004; Hur *et al.*, 2004). Since the concentrations of these cytokines did not increase post-training, it was concluded that the paracrine activity was unaltered by the exercise. However, other factors that were not analysed such as nitric oxide, oxidative stress and MCP-1 might have changed following training. Therefore, future studies should include a wide array of pro and anti-angiogenic molecules in the analysis. Many of the discussion points refer to blood flow profiles and shear stress during exercise as a mechanism for changes following training. Thus, future studies should focus on measuring blood flow during exercise and relate the arterial shear stress patterns to vascular outcomes.

Chapter 7 General Discussion

7.1 Overview of thesis rationale and purpose

The increase in CVD risk throughout the female lifespan due to advancing age and the loss of oestrogen at the menopause is well known. Although CVD prevalence is lower in pre-menopausal women when compared to men, morbidity is very high and is augmented by the presence of CVD risk factors such as obesity. Indeed, global CVD mortality rates are greater in women than men. Despite this, fewer research studies had investigated the effect of interventions on women's health. Therefore, this thesis focused specifically on different populations of women.

A key target for CVD prevention strategies is the vasculature, as endothelial dysfunction and stiffening of the vessel wall accelerates atherosclerosis, the underlying cause of many CVDs. A further mediator of vascular homeostasis involves the contribution of circulating angiogenic cells (CACs) to vascular repair. All of these factors, which worsen throughout the female lifespan, can be improved with exercise training.

The recommended UK government guidelines for exercise participation in adults (≥30 min moderate-intensity exercise on 5 days/week) is very general regarding the type and definition of intensity, and might not be optimal to gain the greatest benefits to vascular health. Additionally, these guidelines may not be applicable to everyone as the optimum exercise prescription may vary between populations (i.e. healthy, male vs. female, obese, heart failure, postmenopausal etc.). Furthermore, exercise adherence rates are very low (4% of women achieve the recommended guidelines) with "a lack of time" and "not

motivated" cited as the two main barriers to exercise participation (British Heart Foundation, 2012). With this in mind, researchers started to explore the impact of different types of exercise on cardiovascular health. Recently, interval exercise has been a focal point of interest as it can be more time-efficient, enjoyable and can exert similar or superior improvements on markers of vascular health, when compared to traditional continuous exercise training methods, and thus, may increase exercise participation rates (Wisloff *et al.*, 2007; Haram *et al.*, 2008; Rakobowchuk *et al.*, 2008; Tjønna *et al.*, 2008). Therefore, this thesis sought to compare the effects of interval and continuous exercise on endothelial function, arterial stiffness and CAC number and function (as markers of vascular health) in young healthy, middle-aged obese and postmenopausal women.

7.2 Design of the exercise sessions

An important mediator of the physiological adaptations to exercise is exercise intensity and should therefore be carefully considered when designing an exercise intervention. Greater physiological stresses exerted at higher exercise intensities have shown to be beneficial for improving markers of vascular health such as endothelial function (Walsh *et al.*, 2003; Rakobowchuk *et al.*, 2008; Silva *et al.*, 2012). Conversely, other studies have reported adverse consequences following high-intensity exercise training with reductions in endothelial function and increases in oxidative stress (Bergholm *et al.*, 1999; Goto *et al.*, 2003). Despite these contrasting results and importance of exercise intensity, the definition of moderate and high-intensity is poorly defined within the literature. The majority of studies normalise intensity to a percentage of $\dot{V}O_{2max}$, HR_{max} or HRR. Although easy to calculate and prescribe, these methods do not accurately control for differences in metabolic stress between

individuals. At a given percentage of $\dot{V}O_{2max}$ or HR_{max}, some individuals may be exercising above their individual lactate threshold, while others can be exercising below and thus, at a different intensity (Meyer *et al.*, 1999; Scharhag-Rosenberger *et al.*, 2010; Rossiter, 2011). Consequently, the physiological and metabolic stress during exercise between individuals would be heterogeneous and hence, may contribute to the discrepancy in vascular health outcomes observed following exercise training in the literature. Additionally, when comparing the effects of different types of exercise such as interval and continuous exercise, it is important to control for the factors that are known to mediate adaptations, in order to determine if one type is better than the other for vascular health improvements. For example, Wisloff *et al*., (2007) observed larger increases in endothelial-dependent vasodilation following interval exercise compared to continuous exercise training. However, interval exercise sessions were likely of a higher intensity than continuous exercise sessions and therefore, the difference in results may actually be mediated by the difference in exercise intensity and not the interval vs. continuous nature of the exercise. Therefore, in this thesis, exercise intensity was either maximal or defined according to the exercise intensity domains, where moderate-intensity is exercise below the individual lactate threshold and heavy-intensity just above the lactate threshold (Rossiter, 2011). A novel aspect from chapters 5 and 6 was that interval and continuous exercise sessions were matched to an intensity domain, in attempt to exclude exercise intensity as a mechanism for any potential differences in outcome between the two exercise types. This allowed the effects of the different work-rate profiles associated with interval and continuous exercise on vascular health to be examined.

7.3 Summary of key findings

In chapter 4, in a population of healthy young females, sprint interval and sprint continuous exercise training similarly increased cardio-respiratory fitness, exercise tolerance and $CD34^{+}$ cell number, but only interval type exercise appeared to change endothelial function, indicated by an increased brachial artery FMD only in the interval exercise training group. It was therefore suggested that the intensity of the exercise might be a more important determinant than the interval vs. continuous nature of the exercise, for increasing cardio-respiratory fitness and mobilisation and survival of haematopoietic progenitor cells, that may aid in endothelial repair or angiogenesis. In contrast, it was hypothesised that the different shear stress profiles experienced by the endothelium during interval and continuous exercise might explain the different outcome in FMD, due to repeated sudden increases in shear stress associated with interval exercise, potentially providing a greater stimulus for endothelial cells. However, in chapter 6 in a population of middle-aged overweight/obese women, heavy-intensity interval exercise training reduced brachial artery FMD, but appeared to increase $CD34^{+}CD45^{dim}$ cell number and CAC adhesive ability, whereas heavy-intensity and work matched continuous exercise training had no effect. Although limited by low participant number in CAC number and function analysis, the results suggest that unlike in a healthy female population where intensity appears to be the most important mediator for CAC release, in a different population of women with presence of CVD risk factors, the interval vs. continuous nature of the exercise influences CAC number and function differently. Likewise, FMD was only altered following interval exercise training, which is similar to the findings from the first study. Even though FMD reduced following interval exercise training, which may or

may not signify a reduction in endothelial function, it still highlights that the fluctuations in work-rate and arterial shear stress during interval exercise, has a different impact on the endothelium than a continuous work-rate and level of shear stress experienced during continuous exercise sessions. In agreement with the first study, cardio-respiratory fitness and exercise tolerance increased following both intensity and work matched interval and continuous exercise training, thus, supporting the theory that the intensity of the exercise mediates improvements in fitness, and not the type of aerobic exercise undertaken.

In chapter 5, the acute effect of moderate-intensity continuous and moderate and heavy-intensity interval exercise on markers of vascular health, was assessed in postmenopausal women. The aim was to determine if interval exercise had a greater immediate impact on vascular health than continuous exercise, and would therefore be more beneficial for postmenopausal women if undertaken chronically. Following the moderate-intensity interval exercise session, reductions in brachial and carotid artery pulse pressure were observed, whilst brachial and carotid artery systolic blood pressure and brachial artery pulse pressure increased following heavy-intensity interval exercise. The opposite results were most likely due to the time point of assessment post-exercise (15 min). Additionally, both moderate and heavy-intensity interval exercise increased the number of CAC colony-forming units post-exercise, whereas moderate-intensity continuous exercise had no effect on any of the variables measured at 15 min post-exercise. The results from this acute exercise study suggest that interval exercise may be more effective than the government recommended guidelines of 30 min of moderate-intensity continuous exercise, for reducing blood pressure and increasing the interactive

ability of angiogenic cells and thus, may partly contribute to preventing the decline in cardiovascular health associated with postmenopausal women.

As a whole, the studies in this thesis found that interval exercise both acutely and chronically, modified more variables related to vascular health in different populations of women than continuous exercise, even when exercise intensity was controlled. Therefore, as illustrated in Figure 7.1, interval exercise may be more effective than continuous exercise in rectifying the imbalance in vascular homeostasis that is associated with advancing age, oestrogen loss at the menopause and obesity, by reducing vascular damage and increasing vascular repair. The results from this thesis support previous research in which interval exercise exerted similar and superior effects on markers of vascular health such as cardio-respiratory fitness and endothelial function (Wisloff *et al.*, 2007; Tjønna *et al.*, 2008; Currie *et al.*, 2013). In addition, the effects on CAC number and function following interval and continuous exercise were compared for the first time, with interval exercise demonstrating similar or superior increases than continuous exercise, especially in CAC function. In contrast, markers of central and peripheral arterial stiffness were not altered following either interval or continuous exercise in any of the studies in this thesis in women across the lifespan. This might be related to good arterial distensibility prior to exercise or an insufficient stimulus for adaptation. Additionally, a higher frequency of exercise sessions may need to be performed each week for reductions in systemic arterial stiffness as observed in previous studies (Sugawara *et al.*, 2006; Guimaraes *et al.*, 2010).

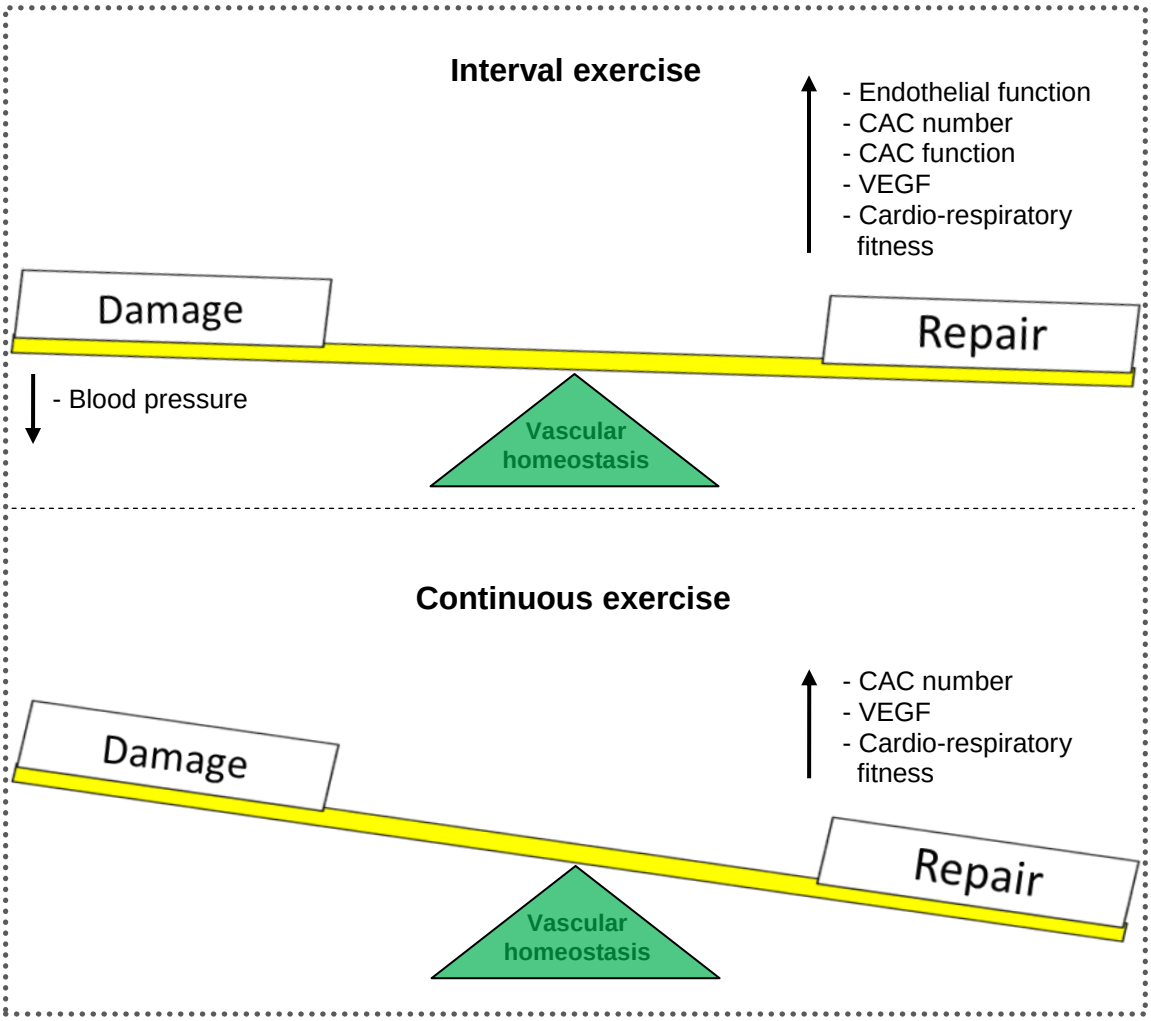

Figure 7.1. The effect of interval and continuous exercise on vascular homeostasis. Given that interval exercise modified more vascular health markers than continuous exercise in this thesis, it is suggested that interval exercise may be more beneficial than continuous exercise in rectifying the imbalance between vascular damage and repair, in populations of women who present risk factors for cardiovascular disease. CAC = circulating angiogenic cell, VEGF = vascular endothelial growth factor.

The combination of examining several markers of vascular health together in each participant following exercise, revealed that exercise does not modify all aspects of vascular health or effect all variables equally, or indeed in the same direction. For example in chapter 6, following interval exercise training, brachial artery FMD decreased but CAC number and function increased. This complex relationship between exercise and markers of vascular health has implications for exercise prescription.

7.4 Implications for exercise prescription in women

The benefits of exercise participation to cardiovascular health is well known and strongly encouraged by the government, physicians and healthcare professionals, in order to reduce the high rates of CVD morbidity and mortality. However, exercise should not be viewed as a “one size fits all” therapy. The results observed in this thesis together with inconsistencies in the literature suggest that exercise prescription should be individualised or specified for different populations. From the studies carried out in this thesis, it was observed that 1) different exercise types (interval vs. continuous) exert different effects on the same variable, 2) exercise has different effects on different markers on vascular health, and 3) the impact of exercise is influenced by the initial vascular health of individuals. Thus, exercise prescription is more complicated than originally perceived. The observation in this thesis that exercise whether studied acutely or chronically, can improve one marker of vascular health such as endothelial function, but have no effect on a different marker such as arterial stiffness, raises the question of how to decide which type and intensity of exercise should be recommended to which population? Potentially, the best course of action is firstly to identify which physiological marker requires the most attention, and when improved with exercise will bring about the greatest reductions to CVD risk. Secondly, the type of exercise that will yield the greatest improvements to the desired variable should be determined and prescribed. For example, in obese women, if the greatest gains in vascular health are achieved by weight loss, then the interval and continuous exercise training programme adopted in chapter 6 should not be prescribed, as participants did not lose weight. Likewise, in a population of hypertensive postmenopausal women, the greatest reductions to CVD risk could be attained through reductions in blood

pressure. Given that a 30 min bout of moderate-intensity interval exercise immediately reduced blood pressure in postmenopausal women in chapter 5, with larger changes observed in individuals with a higher blood pressure at pre-exercise; moderate-intensity interval exercise involving 10 s of work and 20 s of active recovery may be favourable for hypertensive postmenopausal women. Furthermore, for healthy women without risk factors for CVD, exercise participation throughout the lifespan can reduce the increased CVD risk associated with advancing age, by attenuating the decline in endothelial function (DeSouza *et al.*, 2000), CAC number and function (Yang *et al.*, 2013) and arterial compliance (Tanaka *et al.*, 2002), caused by advancing age and the menopause. Given that $\dot{V}O_{2max}$ is one of the main predictors of CVD morbidity and mortality (Laukkanen *et al.*, 2004; Aspenes *et al.*, 2011) and declines with age in women (Fitzgerald *et al.*, 1997), it could be suggested that in healthy women without CVD risk factors, the most important goal in relation to vascular health is the attainment and maintenance of a high level of cardio-respiratory fitness throughout the lifespan. As observed in chapters 4 and 6 in this thesis and in previous studies (Burgomaster *et al.*, 2008; Rakobowchuk *et al.*, 2012), exercise at higher intensities produces greater increases in $\dot{V}O_{2max}$ than lower exercise intensities, which appears to be independent of the type of exercise undertaken. Therefore, an exercise mode which is of a high intensity but short duration may be favourable to healthy women.

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7.5 Limitations and future work

One of the main limitations experienced in chapters 4-6 of this thesis was the reduction in the number of participants that could be included in analysis, due to equipment failure and problems with cell culture. Therefore, it was not possible to draw firm conclusions, especially relating to the effects of exercise on circulating angiogenic cell number and function. Furthermore, the implications of increased CAC number and function on vascular health following exercise is unclear due to the lack of research in this area, and the lack of consensus on what a CAC is and what it does. Moreover, although the culture of CACs allowed the assessment of CAC function, analysis of CACs *in vitro* may not necessarily reflect how the cells behave *in vivo*. Consequently, future studies should focus on isolating CACs from peripheral blood before and after exercise using antibodies, and applying the cells to models of vascular damage *in vivo* such as infusion into ischaemic hind limb of mice.

In the experimental studies in this thesis, several mechanisms for the changes observed following exercise were proposed, including greater nitric oxide bioavailability, reduced oxidative stress, enhanced anti-oxidant enzyme activity, arterial structural changes and exercise session duration. Additionally, one of the main discussion points throughout the thesis was how the differences in haemodynamics and shear stress patterns, between interval and continuous exercise, may have contributed to the divergent results observed. However, these factors were not measured in this thesis. Although the aim of this thesis was not to identify the mechanisms by which interval and continuous exercise modify the variables measured, the addition of assessments of nitric oxide, oxidative stress and in-exercise blood flow would have enhanced the study. Therefore, future studies are required to measure blood flow and shear stress

during interval and continuous exercise, and subsequently apply these profiles to endothelial cells in order to examine the impact on cellular redox state. These studies are necessary to validate the suggestion raised in this thesis that the fluctuations in work-rate and shear stress associated with interval exercise, provides a greater stimulus for enhanced endothelial function.

The interval and continuous exercise protocols adopted in this thesis were chosen to control for exercise intensity. However, this stringent control makes it difficult to translate to the public. In chapter 4, specialised equipment was used and in chapters 5-6, a maximal exercise test was required in order to calculate the exercise session work-rates. Since, nearly all members of the public do not have access to these facilities, future studies should investigate how exercise prescribed using laboratory equipment can be prescribed without the use of any equipment.

7.6 Conclusion

To summarise, the main purpose of the thesis was to compare the effects of interval and continuous exercise on markers of vascular health in different populations of women. For the first time, exercise sessions were controlled to an exercise intensity domain, thereby allowing exercise intensity to be excluded as a mechanism for any differences in outcome between interval and continuous exercise. The data presented indicated that in some variables related to vascular health, interval exercise produced similar or superior effects, which is in agreement with previous studies in the literature. Therefore, interval exercise involving short work:recovery duty cycles may be more beneficial to women across the lifespan than the government recommended guidelines of moderate-intensity continuous exercise, by ameliorating more CVD risk factors.

However, much research is still required. Only three different populations of women were studied, therefore comparisons between the exercise types should be completed in women with different CVD risk factors, or indeed in females who suffer from CVD. Secondly, the effects of the interval exercise protocol used in chapter 6 on endothelial function should be measured in a different population of women and in different arteries, to assess whether the decrease in FMD observed following interval exercise training in overweight/obese women, is reflective of reduced endothelial function or as a result of other mechanisms. Finally, the mechanisms contributing to the different outcomes observed between interval and continuous exercise needs to be elucidated. It is hoped that the mechanism postulated in this thesis relating to the potential different effects that the in-exercise shear stress patterns have on the endothelium during interval and continuous exercise, will be explored in the future.

Chapter 8 References

ACSM. (2006). *ACSM's Guidelines for Exercise Testing and Prescription*. Lippincott Williams & Wilkins Pennsylvania.

Adams V, Lenk K, Linke A, Lenz D, Erbs S, Sandri M, Tarnok A, Gielen S, Emmrich F, Schuler G & Hambrecht R. (2004). Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. *Arteriosclerosis, thrombosis, and vascular biology* **24**, 684-690.

Adams V, Linke A, Kränkel N, Erbs S, Gielen S, Möbius-Winkler S, Gummert JF, Mohr FW, Schuler G & Hambrecht R. (2005). Impact of Regular Physical Activity on the NAD(P)H Oxidase and Angiotensin Receptor System in Patients With Coronary Artery Disease. *Circulation* **111**, 555-562.

Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM & Dimmeler S. (2003). Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* **9**, 1370-1376. .

Aicher A, Zeiher AM & Dimmeler S. (2005). Mobilizing Endothelial Progenitor Cells. *Hypertension* **45**, 321-325.

Akazawa N, Choi Y, Miyaki A, Tanabe Y, Sugawara J, Ajisaka R & Maeda S. (2012). Curcumin ingestion and exercise training improve vascular endothelial function in postmenopausal women. *Nutrition Research* **32**, 795-799.

Alderton WK, Cooper CE & Knowles RG. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochemical Journal* **357**, 593-615.

Allender S, Peto V, Scarborough P, Kaur A & Rayner M. (2008a). Coronary Heart Disease Statistics. British Heart Foundation.

Allender S, Scarborough P, Peto V, Rayner M, Leal J, Luengo-Fernández R & Gray A. (2008b). European Cardiovascular Disease Statistics. ed. GROUP BHFHPR.

Anand SS, Xie CC, Mehta S, Franzosi MG, Joyner C, Chrolavicius S, Fox KAA & Yusuf S. (2005). Differences in the Management and Prognosis of Women and Men Who Suffer From Acute Coronary Syndromes. *Journal of the American College of Cardiology* **46**, 1845-1851.

Anderson TJ, Uehata A, Gerhard MD, Meredith IT, Knab S, Delagrange D, Lieberman EH, Ganz P, Creager MA, Yeung AC & Selwyn AP. (1995). Close relation of endothelial function in the human coronary and peripheral circulations. *Journal of the American College of Cardiology* **26**, 1235-1241.

Armentano R, Megnien JL, Simon A, Bellenfant F, Barra J & Levenson J. (1995). Effects of Hypertension on Viscoelasticity of Carotid and Femoral Arteries in Humans. *Hypertension* **26**, 48-54.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G & Isner JM. (1997). Isolation of Putative Progenitor Endothelial Cells for Angiogenesis. *Science* **275**, 964-966.

Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M & Isner JM. (1999). VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *Embo J* **18**, 3964-3972.

Aspenes ST, Nilsen TIL, Skaug E-A, Bertheussen GF, Ellingsen Ø, Vatten L & Wisløff U. (2011). Peak Oxygen Uptake and Cardiovascular Risk Factors in 4631 Healthy Women and Men. *Medicine & Science in Sports & Exercise* **43**, 1465-1473 1410.1249/MSS.1460b1013e31820ca31881c.

Attipoe S, Park JY, Fenty N, Phares D & Brown M. (2008). Oxidative stress levels are reduced in postmenopausal women with exercise training regardless of hormone replacement therapy status. *J Women Aging* **20**, 31-45.

Aviv H, Yusuf Khan M, Skurnick J, Okuda K, Kimura M, Gardner J, Priolo L & Aviv A. (2001). Age dependent aneuploidy and telomere length of the human vascular endothelium. *Atherosclerosis* **159**, 281-287.

Avogaro A & de Kreutzenberg SV. (2005). Mechanisms of endothelial dysfunction in obesity. *Clinica Chimica Acta* **360**, 9-26.

Bartlett JD, Hwa Joo C, Jeong T-S, Louhelainen J, Cochran AJ, Gibala MJ, Gregson W, Close GL, Drust B & Morton JP. (2012). Matched work high-intensity interval and continuous running induce similar increases in PGC-1α mRNA, AMPK, p38, and p53 phosphorylation in human skeletal muscle. *Journal of Applied Physiology* **112**, 1135-1143.

Beaver WL, Wasserman K & Whipp BJ. (1986). A new method for detecting anaerobic threshold by gas exchange. *Journal of Applied Physiology* **60**, 2020-2027.

Beckman JS, Beckman TW, Chen J, Marshall PA & Freeman BA. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 1620-1624.

Benetos A, Laurent S, Hoeks AP, Boutouyrie PH & Safar ME. (1993). Arterial alterations with aging and high blood pressure. A noninvasive study of carotid and femoral arteries. *Arteriosclerosis, Thrombosis, and Vascular Biology* **13**, 90-97.

Bergholm R, Mäkimattila S, Valkonen M, Liu M-l, Lahdenperä S, Taskinen M-R, Sovijärvi A, Malmberg P & Yki-Järvinen H. (1999). Intense physical training decreases circulating antioxidants and endothelium-dependent vasodilatation in vivo. *Atherosclerosis* **145**, 341-349.

Bertoia ML, Allison MA, Manson JE, Freiberg MS, Kuller LH, Solomon AJ, Limacher MC, Johnson KC, Curb JD, Wassertheil-Smoller S & Eaton CB. (2012). Risk Factors for Sudden Cardiac Death in Post-Menopausal Women. *Journal of the American College of Cardiology*.

Bertovic DA, Waddell TK, Gatzka CD, Cameron JD, Dart AM & Kingwell BA. (1999). Muscular Strength Training Is Associated With Low Arterial Compliance and High Pulse Pressure. *Hypertension* **33**, 1385-1391.

Birk GK, Dawson EA, Atkinson C, Haynes A, Cable NT, Thijssen DH & Green DJ. (2012). Brachial artery adaptation to lower limb exercise training: Role of shear stress. *J Appl Physiol* **8**, 8.

Blacher J, Asmar R, Djane S, London GM & Safar ME. (1999). Aortic Pulse Wave Velocity as a Marker of Cardiovascular Risk in Hypertensive Patients. *Hypertension* **33**, 1111-1117.

Blacher J, Pannier B, Guerin AP, Marchais SJ, Safar ME & London GM. (1998). Carotid Arterial Stiffness as a Predictor of Cardiovascular and All-Cause Mortality in End-Stage Renal Disease. *Hypertension* **32**, 570-574.

Black MA, Cable NT, Thijssen DHJ & Green DJ. (2008). Importance of Measuring the Time Course of Flow-Mediated Dilatation in Humans. *Hypertension* **51**, 203-210.

Bolotina VM, Najibi S, Palacino JJ, Pagano PJ & Cohen RA. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368**, 850-853.

Bonsignore MR, Morici G, Santoro A, Pagano M, Cascio L, Bonanno A, Abate P, Mirabella F, Profita M, Insalaco G, Gioia M, Vignola AM, Majolino I, Testa U & Hogg JC. (2002). Circulating hematopoietic progenitor cells in runners. *Journal of Applied Physiology* **93**, 1691-1697.

Boo YC, Sorescu G, Boyd N, Shiojima I, Walsh K, Du J & Jo H. (2002). Shear Stress Stimulates Phosphorylation of Endothelial Nitric-oxide Synthase at Ser1179 by Akt-independent Mechanisms. *Journal of Biological Chemistry* **277**, 3388-3396.

Boor P, Celec P, Behuliak M, Grančič P, Kebis A, Kukan M, Pronayová N, Liptaj T, Ostendorf T & Šebeková K. (2009). Regular moderate exercise reduces advanced glycation and ameliorates early diabetic nephropathy in obese Zucker rats. *Metabolism* **58**, 1669-1677.

Bots ML, Hoes AW, Koudstaal PJ, Hofman A & Grobbee DE. (1997). Common Carotid Intima-Media Thickness and Risk of Stroke and Myocardial Infarction: The Rotterdam Study. *Circulation* **96**, 1432-1437.

Bousquenaud M, Schwartz C, Léonard F, Rolland-Turner M, Wagner D & Devaux Y. (2012). Monocyte chemotactic protein 3 is a homing factor for circulating angiogenic cells. *Cardiovascular Research*.

Bowen TS, Cannon DT, Begg GA, Baliga V, Witte KK & Rossiter HB. (2012). A novel cardiopulmonary exercise test protocol and criterion to determine maximal oxygen uptake in chronic heart failure. *Journal of Applied Physiology*.

British Heart Foundation. (2010). Coronary heart disease statistics: 2010 edition. In British Heart Foundation Health Promotion Research Group, London.

British Heart Foundation. (2012). Physical Activity Statistics 2012 British Heart Foundation Health Promotion Research Group, London.

Brown M & Wittwer C. (2000). Flow Cytometry: Principles and Clinical Applications in Hematology. *Clinical Chemistry* **46**, 1221-1229.

Bulut D, Albrecht N, Imohl M, Gunesdogan B, Bulut-Streich N, Borgel J, Hanefeld C, Krieg M & Mugge A. (2007). Hormonal status modulates circulating endothelial progenitor cells. *Clin Res Cardiol* **96**, 258-263.

Burgomaster KA, Howarth KR, Phillips SM, Rakobowchuk M, MacDonald MJ, McGee SL & Gibala MJ. (2008). Similar metabolic adaptations during exercise after low volume sprint interval and traditional endurance training in humans. *The Journal of Physiology* **586**, 151-160.

Cai H & Harrison DG. (2000). Endothelial Dysfunction in Cardiovascular Diseases: The Role of Oxidant Stress. *Circulation Research* **87**, 840-844.

Cameron JD & Dart AM. (1994). Exercise training increases total systemic arterial compliance in humans. *American Journal of Physiology - Heart and Circulatory Physiology* **266**, H693-H701.

Cannon DT, White AC, Andriano MF, Kolkhorst FW & Rossiter HB. (2011). Skeletal muscle fatigue precedes the slow component of oxygen uptake kinetics during exercise in humans. *The Journal of Physiology* **589**, 727-739.

Case J, Ingram DA & Haneline LS. (2008). Oxidative Stress Impairs Endothelial Progenitor Cell Function *Antioxid Redox Signal* **10**.

Casey DP, Pierce GL, Howe KS, Mering MC & Braith RW. (2007). Effect of resistance training on arterial wave reflection and brachial artery reactivity in normotensive postmenopausal women. *Eur J Appl Physiol* **100**, 403-408. Epub 2007 Mar 2030.

Celermajer D, Sorensen K, Georgakopoulos D, Bull C, Thomas O, Robinson J & Deanfield J. (1993). Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation* **88**, 2149-2155.

Celermajer DS, Sorensen KE, Gooch VM, Miller, Sullivan ID, Lloyd JK, Deanfield JE & Spiegelhalter DJ. (1992). Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *The Lancet* **340**, 1111-1115.

Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J & Deanfield JE. (1994). Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *Journal of the American College of Cardiology* **24**, 471-476.

Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP & Gurtner GC. (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med* **10**, 858-864. Epub 2004 Jul 2004.

Cesari F, Sofi F, Gori AM, Corsani I, Capalbo A, Caporale R, Abbate R, Gensini GF & Casini A. (2012). Physical activity and circulating endothelial progenitor cells: an intervention study. *European Journal of Clinical Investigation* **42**, 927-932.

Chavakis E, Aicher A, Heeschen C, Sasaki K-i, Kaiser R, El Makhfi N, Urbich C, Peters T, Scharffetter-Kochanek K, Zeiher AM, Chavakis T & Dimmeler S. (2005). Role of β2-integrins for homing and neovascularization capacity of endothelial progenitor cells. *The Journal of Experimental Medicine* **201**, 63-72.

Chen C-H, Ting C-T, Nussbacher A, Nevo E, Kass DA, Pak P, Wang S-P, Chang M-S & Yin FCP. (1996). Validation of Carotid Artery Tonometry as a Means of Estimating Augmentation Index of Ascending Aortic Pressure. *Hypertension* **27**, 168-175.

Chen D-D, Dong Y-G, Yuan H & Chen AF. (2012). Endothelin 1 Activation of Endothelin A Receptor/NADPH Oxidase Pathway and Diminished Antioxidants Critically Contribute to Endothelial Progenitor Cell Reduction and Dysfunction in Salt-Sensitive Hypertension. *Hypertension* **59**, 1037-1043.

Chen Z-P, Mitchelhill KI, Michell BJ, Stapleton D, Rodriguez-Crespo I, Witters LA, Power DA, Ortiz de Montellano PR & Kemp BE. (1999). AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Letters* **443**, 285-289.

Chiu YC, Arand PW, Shroff SG, Feldman T & Carroll JD. (1991). Determination of pulse wave velocities with computerized algorithms. *American Heart Journal* **121**, 1460-1470.

Ciolac EG, Bocchi EA, Bortolotto LA, Carvalho VO, Greve JMD & Guimaraes GV. (2010). Effects of high-intensity aerobic interval training vs. moderate exercise on hemodynamic, metabolic and neuro-humoral abnormalities of young normotensive women at high familial risk for hypertension. *Hypertens Res* **33**, 836-843.

Clopath P, Müller K, Stäubli W & Bürk RR. (1979). *In vivo* and *in vitro* Studies on Endothelial Regeneration. *Pathophysiology of Haemostasis and Thrombosis* **8**, 149-157.

Cocks M, Shaw CS, Shepherd SO, Fisher JP, Ranasinghe AM, Barker TA, Tipton KD & Wagenmakers AJM. (2013). Sprint interval and endurance training are equally effective in increasing muscle microvascular density and eNOS content in sedentary males. *The Journal of Physiology* **591**, 641-656.

Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J & Vogel R. (2002). Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* **39**, 257-265.

Csiszar A, Ungvari Z, Koller A, Edwards JG & Kaley G. (2004). Proinflammatory phenotype of coronary arteries promotes endothelial apoptosis in aging. *Physiological Genomics* **17**, 21-30.

Cubbon RM, Murgatroyd SR, Ferguson C, Bowen TS, Rakobowchuk M, Baliga V, Cannon D, Rajwani A, Abbas A, Kahn M, Birch KM, Porter KE, Wheatcroft SB, Rossiter HB & Kearney MT. (2010). Human Exercise-Induced Circulating Progenitor Cell Mobilization Is Nitric Oxide-Dependent and Is Blunted in South Asian Men. *Arterioscler Thromb Vasc Biol* **30**, 878-884.

Currie KD, Dubberley JB, Mckelvie RS & Macdonald MJ. (2013). Low-Volume, High-Intensity Interval Training in Patients with CAD. *Medicine & Science in Sports & Exercise* **45**, 1436-1442 1410.1249/MSS.1430b1013e31828bbbd31824.

Dai X, Tan Y, Cai S, Xiong X, Wang L, Ye Q, Yan X, Ma K & Cai L. (2011). The role of CXCR7 on the adhesion, proliferation and angiogenesis of endothelial progenitor cells. *Journal of Cellular and Molecular Medicine* **15**, 1299-1309.

Darley-Usmar VM, Hogg N, O'Leary VJ, Wilson MT & Moncada S. (1992). The simultaneous generation of superoxide and nitric oxide can initiate lipid

peroxidation in human low density lipoprotein. *Free Radic Res Commun* **17**, 9-20.

De Filippis E, Cusi K, Ocampo G, Berria R, Buck S, Consoli A & Mandarino LJ. (2006). Exercise-Induced Improvement in Vasodilatory Function Accompanies Increased Insulin Sensitivity in Obesity and Type 2 Diabetes Mellitus. *Journal of Clinical Endocrinology & Metabolism* **91**, 4903-4910.

Dekker MJ, Lee S, Hudson R, Kilpatrick K, Graham TE, Ross R & Robinson LE. (2007). An exercise intervention without weight loss decreases circulating interleukin-6 in lean and obese men with and without type 2 diabetes mellitus. *Metabolism* **56**, 332-338.

DeSouza CA, Shapiro LF, Clevenger CM, Dinenno FA, Monahan KD, Tanaka H & Seals DR. (2000). Regular Aerobic Exercise Prevents and Restores Age-Related Declines in Endothelium-Dependent Vasodilation in Healthy Men. *Circulation* **102**, 1351-1357.

Di Santo S, Diehm N, Ortmann J, Völzmann J, Yang Z, Keo H-H, Baumgartner I & Kalka C. (2008). Oxidized low density lipoprotein impairs endothelial progenitor cell function by downregulation of E-selectin and integrin αvβ5. *Biochemical and Biophysical Research Communications* **373**, 528-532.

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R & Zeiher AM. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**, 601-605.

Dimmeler S & Zeiher AM. (2004). Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis? *Journal of Molecular Medicine* **82**, 671-677.

Donato AJ, Gano LB, Eskurza I, Silver AE, Gates PE, Jablonski K & Seals DR. (2009). Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase. *Am J Physiol-Heart C* **297**, H425-H432.

Doshi SN, Naka KK, Payne N, Jones CJ, Ashton M, Lewis MJ & Goodfellow J. (2001). Flow-mediated dilatation following wrist and upper arm occlusion in humans: the contribution of nitric oxide. *Clin Sci* **101**, 629-635.

Dusserre N, L'Heureux N, Bell KS, Stevens HY, Yeh J, Otte LA, Loufrani L & Frangos JA. (2004). PECAM-1 interacts with nitric oxide synthase in human endothelial cells: implication for flow-induced nitric oxide synthase activation. *Arteriosclerosis, Thrombosis & Vascular Biology* **24**, 1796-1802.

Edwards DG, Schofield RS, Lennon SL, Pierce GL, Nichols WW & Braith RW. (2004). Effect of exercise training on endothelial function in men with coronary artery disease. *The American Journal of Cardiology* **93**, 617-620.

Egashira K, Inou T, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Kuga T, Urabe Y & Takeshita A. (1993). Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans. *Circulation* **88**, 77-81.

Egginton S, Badr I, Williams J, Hauton D, Baan GC & Jaspers RT. (2011). Physiological angiogenesis is a graded, not threshold, response. *The Journal of Physiology* **589**, 195-206.

Endtmann C, Ebrahimian T, Czech T, Arfa O, Laufs U, Fritz M, Wassmann K, Werner N, Petoumenos V, Nickenig G & Wassmann S. (2011). Angiotensin II Impairs Endothelial Progenitor Cell Number and Function In Vitro and In Vivo. *Hypertension* **58**, 394-403.

Erbs S, Höllriegel R, Linke A, Beck EB, Adams V, Gielen S, Möbius-Winkler S, Sandri M, Kränkel N, Hambrecht R & Schuler G. (2010). Exercise Training in Patients With Advanced Chronic Heart Failure (NYHA IIIb) Promotes Restoration of Peripheral Vasomotor Function, Induction of Endogenous Regeneration, and Improvement of Left Ventricular Function. *Circulation: Heart Failure* **3**, 486-494.

Erusalimsky JD. (2009). Vascular endothelial senescence: from mechanisms to pathophysiology. *Journal of Applied Physiology* **106**, 326-332.

Esposito K, Ciotola M, Schisano B, Gualdiero R, Sardelli L, Misso L, Giannetti G & Giugliano D. (2006). Endothelial Microparticles Correlate with Endothelial Dysfunction in Obese Women. *Journal of Clinical Endocrinology & Metabolism* **91**, 3676-3679.

Fadini GP, Coracina A, Baesso I, Agostini C, Tiengo A, Avogaro A & Vigili de Kreutzenberg S. (2006a). Peripheral Blood CD34+KDR+ Endothelial Progenitor Cells Are Determinants of Subclinical Atherosclerosis in a Middle-Aged General Population. *Stroke* **37**, 2277-2282.

Fadini GP, de Kreutzenberg S, Albiero M, Coracina A, Pagnin E, Baesso I, Cignarella A, Bolego C, Plebani M, Nardelli GB, Sartore S, Agostini C & Avogaro A. (2008). Gender Differences in Endothelial Progenitor Cells and Cardiovascular Risk Profile: The Role of Female Estrogens. *Arterioscler Thromb Vasc Biol* **28**, 997-1004.

Fadini GP, de Kreutzenberg SV, Coracina A, Baesso I, Agostini C, Tiengo A & Avogaro A. (2006b). Circulating CD34+ cells, metabolic syndrome, and cardiovascular risk. *European Heart Journal* **27**, 2247-2255.

Fadini GP, Sartore S, Albiero M, Baesso I, Murphy E, Menegolo M, Grego F, Vigili de Kreutzenberg S, Tiengo A, Agostini C & Avogaro A. (2006c). Number and Function of Endothelial Progenitor Cells as a Marker of Severity for Diabetic Vasculopathy. *Arteriosclerosis, Thrombosis, and Vascular Biology* **26**, 2140-2146.

Farrar DJ, Bond MG, Sawyer JK & Green HD. (1984). Pulse wave velocity and morphological changes associated with early atherosclerosis progression in the aortas of cynomolgus monkeys. *Cardiovascular Research* **18**, 107-118.

Fernandes T, Nakamuta JS, Magalhães FC, Roque FR, Lavini-Ramos C, Schettert IT, Coelho V, Krieger JE & Oliveira EM. (2012). Exercise training restores the endothelial progenitor cells number and function in hypertension: implications for angiogenesis. *Journal of Hypertension* **30**, 2133-2143 2110.1097/HJH.2130b2013e3283588d3283546.

Ferrier KE, Waddell TK, Gatzka CD, Cameron JD, Dart AM & Kingwell BA. (2001). Aerobic Exercise Training Does Not Modify Large-Artery Compliance in Isolated Systolic Hypertension. *Hypertension* **38**, 222-226.

Field A. (2013). *Discovering statistics using IBM SPSS statistics*. SAGE Publications Ltd, London.

Fitzgerald MD, Tanaka H, Tran ZV & Seals DR. (1997). Age-related declines in maximal aerobic capacity in regularly exercising vs. sedentary women: a meta-analysis. *Journal of Applied Physiology* **83**, 160-165.

Fleenor BS, Marshall KD, Durrant JR, Lesniewski LA & Seals DR. (2010). Arterial stiffening with ageing is associated with transforming growth factor-β1-related changes in adventitial collagen: reversal by aerobic exercise. *The Journal of Physiology* **588**, 3971-3982.

Förstermann U & Münzel T. (2006). Endothelial Nitric Oxide Synthase in Vascular Disease: From Marvel to Menace. *Circulation* **113**, 1708-1714.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD & Semenza GL. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Molecular and Cellular Biology* **16**, 4604-4613.

Franklin SS, Khan SA, Wong ND, Larson MG & Levy D. (1999). Is Pulse Pressure Useful in Predicting Risk for Coronary Heart Disease?: The Framingham Heart Study. *Circulation* **100**, 354-360.

Fuchsjäger-Mayrl G, Pleiner J, Wiesinger GF, Sieder AE, Quittan M, Nuhr MJ, Francesconi C, Seit H-P, Francesconi M, Schmetterer L & Wolzt M. (2002). Exercise Training Improves Vascular Endothelial Function in Patients with Type 1 Diabetes. *Diabetes Care* **25**, 1795-1801.

Fujiyama S, Amano K, Uehira K, Yoshida M, Nishiwaki Y, Nozawa Y, Jin D, Takai S, Miyazaki M, Egashira K, Imada T, Iwasaka T & Matsubara H. (2003). Bone Marrow Monocyte Lineage Cells Adhere on Injured Endothelium in a Monocyte Chemoattractant Protein-1–Dependent Manner and Accelerate Reendothelialization as Endothelial Progenitor Cells. *Circulation Research* **93**, 980-989.

Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G & Harrison DG. (2000). Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *The Journal of Clinical Investigation* **105**, 1631-1639.

Furchgott RF & Zawadzki JV. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* **288**, 373-376.

Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M & Shimomura I. (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. *The Journal of Clinical Investigation* **114**, 1752-1761.

Gatta L, Armani A, Iellamo F, Consoli C, Molinari F, Caminiti G, Volterrani M & Rosano GMC. (2012). Effects of a short-term exercise training on serum factors involved in ventricular remodelling in chronic heart failure patients. *International Journal of Cardiology* **155**, 409-413.

George J, Herz I, Goldstein E, Abashidze S, Deutch V, Finkelstein A, Michowitz Y, Miller H & Keren G. (2003). Number and Adhesive Properties of Circulating Endothelial Progenitor Cells in Patients With In-Stent Restenosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **23**, e57-e60.

Gerrits H, van Ingen Schenau DS, Bakker NEC, van Disseldorp AJM, Strik A, Hermens LS, Koenen TB, Krajnc-Franken MAM & Gossen JA. (2008). Early postnatal

lethality and cardiovascular defects in CXCR7-deficient mice. *genesis* **46**, 235-245.

Gibala MJ, Little JP, van Essen M, Wilkin GP, Burgomaster KA, Safdar A, Raha S & Tarnopolsky MA. (2006). Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. *The Journal of Physiology* **575**, 901-911.

Gielen S, Schuler G & Adams V. (2010). Cardiovascular Effects of Exercise Training. *Circulation* **122**, 1221-1238.

Glasser SP, Arnett DK, McVeigh GE, Finkelstein SM, Bank AJ, Morgan DJ & Cohn JN. (1997). Vascular Compliance and Cardiovascular Disease: A Risk Factor or a Marker? *American Journal of Hypertension* **10**, 1175-1189.

Gomes EC, Silva AN & de Oliveira MR. (2012). Oxidants, antioxidants, and the beneficial roles of exercise-induced production of reactive species. *Oxid Med Cell Longev* **2012**, 756132. Epub 752012 Jun 756133.

Goto C, Higashi Y, Kimura M, Noma K, Hara K, Nakagawa K, Kawamura M, Chayama K, Yoshizumi M & Nara I. (2003). Effect of Different Intensities of Exercise on Endothelium-Dependent Vasodilation in Humans: Role of Endothelium-Dependent Nitric Oxide and Oxidative Stress. *Circulation* **108**, 530-535.

Gragasin FS, Xu Y, Arenas IA, Kainth N & Davidge ST. (2003). Estrogen Reduces Angiotensin II-Induced Nitric Oxide Synthase and NAD(P)H Oxidase Expression in Endothelial Cells. *Arterioscler Thromb Vasc Biol* **23**, 38-44.

Green DJ, Jones H, Thijssen D, Cable NT & Atkinson G. (2011). Flow-Mediated Dilation and Cardiovascular Event Prediction. *Hypertension* **57**, 363-369.

Griendling KK, Sorescu D & Ushio-Fukai M. (2000). NAD(P)H Oxidase : Role in Cardiovascular Biology and Disease. *Circ Res* **86**, 494-501.

Guimaraes GV, Ciolac EG, Carvalho VO, D'Avila VM, Bortolotto LA & Bocchi EA. (2010). Effects of continuous vs. interval exercise training on blood pressure and arterial stiffness in treated hypertension. *Hypertens Res* **33**, 627-632. doi: 610.1038/hr.2010.1042. Epub 2010 Apr 1039.

Gustafsson T, Puntschart A, Kaijser L, Jansson E & Sundberg CJ. (1999). Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. *American Journal of Physiology - Heart and Circulatory Physiology* **276**, H679-H685.

Hagensen MK, Raarup MK, Mortensen MB, Thim T, Nyengaard JR, Falk E & Bentzon JF. (2012). Circulating endothelial progenitor cells do not contribute to regeneration of endothelium after murine arterial injury. *Cardiovascular Research* **93**, 223-231.

Halcox JPJ, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, Nour KRA & Quyyumi AA. (2002). Prognostic Value of Coronary Vascular Endothelial Dysfunction. *Circulation* **106**, 653-658.

Halliwill JR, Buck TM, Lacewell AN & Romero SA. (2013). Postexercise hypotension and sustained postexercise vasodilatation: what happens after we exercise? *Experimental Physiology* **98**, 7-18.

Hamada H, Kim MK, Iwakura A, Ii M, Thorne T, Qin G, Asai J, Tsutsumi Y, Sekiguchi H, Silver M, Wecker A, Bord E, Zhu Y, Kishore R & Losordo DW. (2006). Estrogen Receptors {alpha} and {beta} Mediate Contribution of Bone Marrow-Derived Endothelial Progenitor Cells to Functional Recovery After Myocardial Infarction. *Circulation* **114**, 2261-2270.

Hambrecht R, Adams V, Erbs S, Linke A, Krankel N, Shu Y, Baither Y, Gielen S, Thiele H, Gummert JF, Mohr FW & Schuler G. (2003). Regular Physical Activity Improves Endothelial Function in Patients With Coronary Artery Disease by Increasing Phosphorylation of Endothelial Nitric Oxide Synthase. *Circulation* **107**, 3152-3158.

Hamilton CA, Brosnan MJ, McIntyre M, Graham D & Dominiczak AF. (2001). Superoxide Excess in Hypertension and Aging : A Common Cause of Endothelial Dysfunction. *Hypertension* **37**, 529-534.

Haram PM, Kemi OJ, Lee SJ, Bendheim MØ, Al-Share QY, Waldum HL, Gillian LJ, Koch LG, Britton SL, Najjar SM & Wisløff U. (2008). Aerobic interval training vs. continuous moderate exercise in the metabolic syndrome of rats artificially selected for low aerobic capacity. *Cardiovascular Research*.

Harris RA, Nishiyama SK, Wray DW & Richardson RS. (2010). Ultrasound Assessment of Flow-Mediated Dilation. *Hypertension* **55**, 1075-1085.

Harris RA, Padilla J, Hanlon KP, Rink LD & Wallace JP. (2008). The Flow-mediated Dilation Response to Acute Exercise in Overweight Active and Inactive Men. *Obesity* **16**, 578-584.

Harvey PJ, Morris BL, Kubo T, Picton PE, Su WS, Notarius CF & Floras JS. (2005). Hemodynamic after-effects of acute dynamic exercise in sedentary normotensive postmenopausal women. *Journal of Hypertension* **23**, 285-292.

Hattori K, Heissig B, Tashiro K, Honjo T, Tateno M, Shieh J-H, Hackett NR, Quitoriano MS, Crystal RG, Rafii S & Moore MAS. (2001). Plasma elevation of stromal cell–derived factor-1 induces mobilization of mature and immature hematopoietic progenitor and stem cells. *Blood* **97**, 3354-3360.

Haynes MP, Sinha D, Russell KS, Collinge M, Fulton D, Morales-Ruiz M, Sessa WC & Bender JR. (2000). Membrane Estrogen Receptor Engagement Activates Endothelial Nitric Oxide Synthase via the PI3-Kinase-Akt Pathway in Human Endothelial Cells. *Circ Res* **87**, 677-682.

Hayward CS & Kelly RP. (1997). Gender-Related Differences in the Central Arterial Pressure Waveform. *Journal of the American College of Cardiology* **30**, 1863-1871.

He T, Joyner MJ & Katusic ZS. (2009). Aging decreases expression and activity of glutathione peroxidase-1 in human endothelial progenitor cells. *Microvascular Research* **78**, 447-452.

Heida N-M, Müller J-P, Cheng IF, Leifheit-Nestler M, Faustin V, Riggert J, Hasenfuss G, Konstantinides S & Schäfer K. (2010). Effects of Obesity and Weight Loss on the Functional Properties of Early Outgrowth Endothelial Progenitor Cells. *Journal of the American College of Cardiology* **55**, 357-367.

Heiss C, Keymel S, Niesler U, Ziemann J, Kelm M & Kalka C. (2005). Impaired Progenitor Cell Activity in Age-Related Endothelial Dysfunction. *Journal of the American College of Cardiology* **45**, 1441-1448.

Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MAS, Werb Z & Rafii S. (2002). Recruitment of Stem and Progenitor Cells from the Bone Marrow Niche Requires MMP-9 Mediated Release of Kit-Ligand. *Cell* **109**, 625-637.

Henriksson P, Stamberger M, Eriksson M, Rudling M, Diczfalusy U, Berglund L & Angelin B. (1989). Oestrogen-induced changes in lipoprotein metabolism: role in prevention of atherosclerosis in the cholesterol-fed rabbit. *European Journal of Clinical Investigation* **19**, 395-403.

Henry RMA, Ferreira I, Kostense PJ, Dekker JM, Nijpels G, Heine RJ, Kamp O, Bouter LM & Stehouwer CDA. (2004). Type 2 diabetes is associated with impaired endothelium-dependent, flow-mediated dilation, but impaired glucose metabolism is not: The Hoorn Study. *Atherosclerosis* **174**, 49-56.

Herr MD, Hogeman CS, Koch DW, Krishnan A, Momen A & Leuenberger UA. (2010). A real-time device for converting Doppler ultrasound audio signals into fluid flow velocity. *American Journal of Physiology - Heart and Circulatory Physiology* **298**, H1626-H1632.

Higashi Y, Sasaki S, Kurisu S, Yoshimizu A, Sasaki N, Matsuura H, Kajiyama G & Oshima T. (1999). Regular Aerobic Exercise Augments Endothelium-Dependent Vascular Relaxation in Normotensive As Well As Hypertensive Subjects: Role of Endothelium-Derived Nitric Oxide. *Circulation* **100**, 1194-1202.

Hill JM, Zalos G, Halcox JPJ, Schenke WH, Waclawiw MA, Quyyumi AA & Finkel T. (2003). Circulating Endothelial Progenitor Cells, Vascular Function, and Cardiovascular Risk. *New England Journal of Medicine* **348**, 593-600.

Hirschi KK, Ingram DA & Yoder MC. (2008). Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**, 1584-1595. Epub 2008 Jul 1531.

Hoetzer GL, Van Guilder GP, Irmiger HM, Keith RS, Stauffer BL & DeSouza CA. (2007). Aging, exercise, and endothelial progenitor cell clonogenic and migratory capacity in men. *Journal of Applied Physiology* **102**, 847-852.

Hoffmann J, Haendeler J, Aicher A, Rössig L, Vasa M, Zeiher AM & Dimmeler S. (2001). Aging Enhances the Sensitivity of Endothelial Cells Toward Apoptotic Stimuli: Important Role of Nitric Oxide. *Circulation Research* **89**, 709-715.

Hu CH, Ke X, Chen K, Yang DY, Du ZM & Wu GF. (2013). Transplantation of human umbilical cord-derived endothelial progenitor cells promotes re-endothelialization of the injured carotid artery after balloon injury in New Zealand white rabbits. *Chin Med J (Engl)* **126**, 1480-1485.

Huang P-H, Chen Y-H, Chen Y-L, Wu T-C, Chen J-W & Lin S-J. (2007). Vascular endothelial function and circulating endothelial progenitor cells in patients with cardiac syndrome X. *Heart* **93**, 1064-1070.

Hur J, Yoon C-H, Kim H-S, Choi J-H, Kang H-J, Hwang K-K, Oh B-H, Lee M-M & Park Y-B. (2004). Characterization of Two Types of Endothelial Progenitor Cells and

Their Different Contributions to Neovasculogenesis. *Arterioscler Thromb Vasc Biol* **24**, 288-293.

Imanishi R, Seto S, Toda G, Yoshida M, Ohtsuru A, Koide Y, Baba T & Yano K. (2004a). High brachial-ankle pulse wave velocity is an independent predictor of the presence of coronary artery disease in men. *Hypertension research : official journal of the Japanese Society of Hypertension* **27**, 71-78.

Imanishi T, Hano T & Nishio I. (2005a). Angiotensin II accelerates endothelial progenitor cell senescence through induction of oxidative stress. *Journal of Hypertension* **23**, 97-104.

Imanishi T, Hano T & Nishio I. (2005b). Estrogen reduces angiotensin II-induced acceleration of senescence in endothelial progenitor cells. *Hypertens Res* **28**, 263-271.

Imanishi T, Hano T & Nishio I. (2005c). Estrogen reduces endothelial progenitor cell senescence through augmentation of telomerase activity. *Journal of Hypertension* **23**, 1699-1706.

Imanishi T, Hano T, Sawamura T & Nishio I. (2004b). Oxidized low-density lipoprotein induces endothelial progenitor cell senescence, leading to cellular dysfunction. *Clinical and Experimental Pharmacology and Physiology* **31**, 407-413.

Imanishi T, Tsujioka H & Akasaka T. (2010). Endothelial progenitor cell senescence is there a role for estrogen? *Therapeutic Advances in Cardiovascular Disease* **4**, 55-69.

Iwakura A, Luedemann C, Shastry S, Hanley A, Kearney M, Aikawa R, Isner JM, Asahara T & Losordo DW. (2003). Estrogen-Mediated, Endothelial Nitric Oxide Synthase-Dependent Mobilization of Bone Marrow-Derived Endothelial Progenitor Cells Contributes to Reendothelialization After Arterial Injury. *Circulation* **108**, 3115-3121.

Jacob MP. (2003). Extracellular matrix remodeling and matrix metalloproteinases in the vascular wall during aging and in pathological conditions. *Biomedicine & Pharmacotherapy* **57**, 195-202.

Jenkins NT, Landers RQ, Prior SJ, Soni N, Spangenburg EE & Hagberg JM. (2011). Effects of acute and chronic endurance exercise on intracellular nitric oxide and superoxide in circulating CD34 and CD34 cells. *J Appl Physiol* **111**, 929-937. Epub 2011 Jun 2023.

Jenkins NT, Padilla J, Boyle LJ, Credeur DP, Laughlin MH & Fadel PJ. (2013). Disturbed Blood Flow Acutely Induces Activation and Apoptosis of the Human Vascular Endothelium. *Hypertension* **61**, 615-621.

Jialal I, Devaraj S, Singh U & Huet BA. (2010a). Decreased number and impaired functionality of endothelial progenitor cells in subjects with metabolic syndrome: Implications for increased cardiovascular risk. *Atherosclerosis* **211**, 297-302.

Jialal I, Fadini GP, Pollock K & Devaraj S. (2010b). Circulating Levels of Endothelial Progenitor Cell Mobilizing Factors in the Metabolic Syndrome. *The American Journal of Cardiology* **106**, 1606-1608.

Joint Health Surveys Unit. (2008). Health Survey for England 2007: Healthy lifestyles: knowledge, attitudes and behaviour. ed. THE STATIONERY OFFICE, London.

Joyner MJ. (2000). Effect of Exercise on Arterial Compliance. *Circulation* **102**, 1214-1215.

Jungersten L, Ambring A, Wall B & Wennmalm Å. (1997). Both physical fitness and acute exercise regulate nitric oxide formation in healthy humans. *Journal of Applied Physiology* **82**, 760-764.

Kadoglou NPE, Iliadis F, Angelopoulou N, Perrea D, Ampatzidis G, Liapis CD & Alevizos M. (2007). The anti-inflammatory effects of exercise training in patients with type 2 diabetes mellitus. *European Journal of Cardiovascular Prevention & Rehabilitation* **14**, 837-843.

Kass DA, Shapiro EP, Kawaguchi M, Capriotti AR, Scuteri A, deGroof RC & Lakatta EG. (2001). Improved Arterial Compliance by a Novel Advanced Glycation End-Product Crosslink Breaker. *Circulation* **104**, 1464-1470.

Katz SD, Yuen J, Bijou R & Lejemtel TH. (1997). Training improves endothelium-dependent vasodilation in resistance vessels of patients with heart failure. *Journal of Applied Physiology* **82**, 1488-1492.

Kawasaki T, Sasayama S, Yagi S-I, Asakawa T & Hirai T. (1987). Non-invasive assessment of the age related changes in stiffness of major branches of the human arteries. *Cardiovascular Research* **21**, 678-687.

Kelly R, Hayward C, Avolio A & O'Rourke M. (1989a). Noninvasive determination of age-related changes in the human arterial pulse. *Circulation* **80**, 1652-1659.

Kelly R, Hayward C, Ganis J, Daley J, Avolio A & O'Rourke M. (1989b). Noninvasive registration of the arterial pressure pulse waveform using high-fidelity applanation tonometry. *J Vasc Med Biol* **1**, 142-149.

Keteyian SJ, Brawner CA, Savage PD, Ehrman JK, Schairer J, Divine G, Aldred H, Ophaug K & Ades PA. (2008). Peak aerobic capacity predicts prognosis in patients with coronary heart disease. *American Heart Journal* **156**, 292-300.

Kingwell BA, Sherrard B, Jennings GL & Dart AM. (1997). Four weeks of cycle training increases basal production of nitric oxide from the forearm. *American Journal of Physiology - Heart and Circulatory Physiology* **272**, H1070-H1077.

Kobayashi N, Tsuruya Y, Iwasawa T, Ikeda N, Hashimoto S, Yasu T, Ueba H, Kubo N, Fujii M, Kawakami M & Saito M. (2003). Exercise Training in Patients With Chronic Heart Failure Improves Endothelial Function Predominantly in the Trained Extremities. *Circulation Journal* **67**, 505-510.

Kojda G & Hambrecht R. (2005). Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? *Cardiovascular Research* **67**, 187-197.

Kubes P, Suzuki M & Granger DN. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci U S A* **88**, 4651-4655.

Kuchan MJ & Frangos JA. (1994). Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. *American Journal of Physiology - Cell Physiology* **266**, C628-C636.

Kushner EJ, MacEneaney OJ, Weil BR, Greiner JJ, Stauffer BL & DeSouza CA. (2011). Aging Is Associated with a Proapoptotic Endothelial Progenitor Cell Phenotype. *Journal of Vascular Research* **48**, 408-414.

Kushner EJ, Van Guilder GP, Maceneaney OJ, Cech JN, Stauffer BL & DeSouza CA. (2009). Aging and endothelial progenitor cell telomere length in healthy men. *Clin Chem Lab Med* **47**, 47-50. doi: 10.1515/CCLM.2009.1016.

Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, Mitch WE & Harrison DG. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *The Journal of Clinical Investigation* **111**, 1201-1209.

Landmesser U, Hornig B & Drexler H. (2004). Endothelial Function: A Critical Determinant in Atherosclerosis? *Circulation* **109**, II-27-33.

Lapidot T & Petit I. (2002). Current understanding of stem cell mobilization: The roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Experimental Hematology* **30**, 973-981.

Laufs U, Urhausen A, Werner N, Scharhag J, Heitz A, Kissner G, Böhm M, Kindermann W & Nickenig G. (2005). Running exercise of different duration and intensity: effect on endothelial progenitor cells in healthy subjects. *Journal of Cardiovascular Risk* **12**, 407-414.

Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M & Nickenig G. (2004). Physical Training Increases Endothelial Progenitor Cells, Inhibits Neointima Formation, and Enhances Angiogenesis. *Circulation* **109**, 220-226.

Laughlin MH, Newcomer SC & Bender SB. (2008). Importance of hemodynamic forces as signals for exercise-induced changes in endothelial cell phenotype. *Journal of Applied Physiology* **104**, 588-600.

Laughlin MH, Woodman CR, Schrage WG, Gute D & Price EM. (2004). Interval sprint training enhances endothelial function and eNOS content in some arteries that perfuse white gastrocnemius muscle. *Journal of Applied Physiology* **96**, 233-244.

Laukkanen JA, Kurl S, Salonen R, Rauramaa R & Salonen JT. (2004). The predictive value of cardiorespiratory fitness for cardiovascular events in men with various risk profiles: a prospective population-based cohort study. *European Heart Journal* **25**, 1428-1437.

Lavrenčič Aa, Salobir BGi & Keber I. (2000). Physical Training Improves Flow-Mediated Dilation in Patients With the Polymetabolic Syndrome. *Arteriosclerosis, Thrombosis, and Vascular Biology* **20**, 551-555.

Lerner DJ & Kannel WB. (1986). Patterns of coronary heart disease morbidity and mortality in the sexes: A 26-year follow-up of the Framingham population. *American Heart Journal* **111**, 383-390.

Li D, Saldeen T, Romeo F & Mehta JL. (2000). Oxidized LDL Upregulates Angiotensin II Type 1 Receptor Expression in Cultured Human Coronary Artery Endothelial Cells : The Potential Role of Transcription Factor NF-{kappa}B. *Circulation* **102**, 1970-1976.

Lieberman EH, Gerhard MD, Uehata A, Walsh BW, Selwyn AP, Ganz P, Yeung AC & Creager MA. (1994). Estrogen Improves Endothelium-Dependent, Flow-Mediated Vasodilation in Postmenopausal Women. *Annals of Internal Medicine* **121**, 936-941.

Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, Ford E, Furie K, Go A, Greenlund K, Haase N, Hailpern S, Ho M, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott M, Meigs J, Mozaffarian D, Nichol G, O'Donnell C, Roger V, Rosamond W, Sacco R, Sorlie P, Stafford R, Steinberger J, Thom T, Wasserthiel-Smoller S, Wong N, Wylie-Rosett J, Hong Y, Committee ftAHAS & Subcommittee SS. (2009). Heart Disease and Stroke Statistics—2009 Update: A Report From the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **119**, e21-e181.

London GM, Guerin AP, Pannier B, Marchais SJ & Stimpel M. (1995). Influence of Sex on Arterial Hemodynamics and Blood Pressure: Role of Body Height. *Hypertension* **26**, 514-519.

Losordo DW, Kearney M, Kim EA, Jekanowski J & Isner JM. (1994). Variable expression of the estrogen receptor in normal and atherosclerotic coronary arteries of premenopausal women. *Circulation* **89**, 1501-1510.

Luk T-H, Dai Y-L, Siu C-W, Yiu K-H, Chan H-T, Lee SW, Li S-W, Fong B, Wong W-K, Tam S, Lau C-P & Tse H-F. (2012). Effect of exercise training on vascular endothelial function in patients with stable coronary artery disease: a randomized controlled trial. *European Journal of Preventive Cardiology* **19**, 830-839.

Lund AK. (2010). Oxidants and Endothelial Dysfunction. In *Comprehensive Toxicology*. ed. CHARLENE AM, pp. 243-274. Elsevier, Oxford.

Lusis AJ. (2000). Atherosclerosis. *Nature* **407**, 233-241.

Maas AH & Appelman YE. (2010). Gender differences in coronary heart disease. *Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation* **18**, 598-602.

MacDougall JD, Hicks AL, MacDonald JR, McKelvie RS, Green HJ & Smith KM. (1998). Muscle performance and enzymatic adaptations to sprint interval training. *Journal of Applied Physiology* **84**, 2138-2142.

MacEneaney OJ, Kushner EJ, Van Guilder GP, Greiner JJ, Stauffer BL & DeSouza CA. (2009). Endothelial progenitor cell number and colony-forming capacity in overweight and obese adults. *Int J Obes* **33**, 219-225.

MacEneaney OJ, Kushner EJ, Westby CM, Cech JN, Greiner JJ, Stauffer BL & DeSouza CA. (2010). Endothelial Progenitor Cell Function, Apoptosis, and Telomere Length in Overweight/Obese Humans. *Obesity* **18**, 1677-1682.

Maiorana A, O'Driscoll G, Dembo L, Cheetham C, Goodman C, Taylor R & Green D. (2000). Effect of aerobic and resistance exercise training on vascular function in heart failure. *American Journal of Physiology - Heart and Circulatory Physiology* **279**, H1999-H2005.

Makin AJ, Blann AD, Chung NAY, Silverman SH & Lip GYH. (2004). Assessment of endothelial damage in atherosclerotic vascular disease by quantification of

circulating endothelial cells: Relationship with von Willebrand factor and tissue factor. *European Heart Journal* **25**, 371-376.

Marti CN, Gheorghiade M, Kalogeropoulos AP, Georgiopoulou VV, Quyyumi AA & Butler J. (2012). Endothelial Dysfunction, Arterial Stiffness, and Heart Failure. *Journal of the American College of Cardiology* **60**, 1455-1469.

Matsuda M, Nosaka T, Sato M & Ohshima N. (1993). Effects of physical exercise on the elasticity and elastic components of the rat aorta.

McDermott MM, Ades P, Guralnik JM & et al. (2009). Treadmill exercise and resistance training in patients with peripheral arterial disease with and without intermittent claudication: A randomized controlled trial. *JAMA* **301**, 165-174.

McEleavy OD, McCallum RW, Petrie JR, Small M, Connell JMC, Sattar N & Cleland SJ. (2004). Higher carotid-radial pulse wave velocity in healthy offspring of patients with Type 2 diabetes. *Diabetic Medicine* **21**, 262-266.

McEniery CM, Wallace S, Mackenzie IS, McDonnell B, Yasmin, Newby DE, Cockcroft JR & Wilkinson IB. (2006). Endothelial Function Is Associated With Pulse Pressure, Pulse Wave Velocity, and Augmentation Index in Healthy Humans. *Hypertension* **48**, 602-608.

McNeilly AM, McClean C, Murphy M, McEneny J, Trinick T, Burke G, Duly E, McLaughlin J & Davison G. (2012). Exercise training and impaired glucose tolerance in obese humans. *Journal of Sports Sciences* **30**, 725-732.

McNulty M, Mahmud A & Feely J. (2007). Advanced Glycation End-Products and Arterial Stiffness in Hypertension*. *American Journal of Hypertension* **20**, 242-247.

McVeigh GE, Bratteli CW, Morgan DJ, Alinder CM, Glasser SP, Finkelstein SM & Cohn JN. (1999). Age-Related Abnormalities in Arterial Compliance Identified by Pressure Pulse Contour Analysis: Aging and Arterial Compliance. *Hypertension* **33**, 1392-1398.

Meaume S, Benetos A, Henry OF, Rudnichi A & Safar ME. (2001). Aortic Pulse Wave Velocity Predicts Cardiovascular Mortality in Subjects >70 Years of Age. *Arteriosclerosis, Thrombosis, and Vascular Biology* **21**, 2046-2050.

Mestek ML, Westby CM, Van Guilder GP, Greiner JJ, Stauffer BL & DeSouza CA. (2010). Regular Aerobic Exercise, Without Weight Loss, Improves Endothelium-dependent Vasodilation in Overweight and Obese Adults. *Obesity* **18**, 1667-1669.

Meyer T, Gabriel HH & Kindermann W. (1999). Is determination of exercise intensities as percentages of VO2max or HRmax adequate? *Med Sci Sports Exerc* **31**, 1342-1345.

Michell BJ, Griffiths JE, Mitchelhill KI, Rodriguez-Crespo I, Tiganis T, Bozinovski S, de Montellano PRO, Kemp BE & Pearson RB. (1999). The Akt kinase signals directly to endothelial nitric oxide synthase. *Current biology : CB* **9**, 845.

Miller-Kasprzak E, Bogdański P, Pupek-Musialik D & Jagodziński PP. (2011). Insulin Resistance and Oxidative Stress Influence Colony-Forming Unit-Endothelial Cells Capacity in Obese Patients. *Obesity* **19**, 736-742.

Milstien S & Katusic Z. (1999). Oxidation of Tetrahydrobiopterin by Peroxynitrite: Implications for Vascular Endothelial Function. *Biochemical and Biophysical Research Communications* **263**, 681-684.

Mitchell GF, Hwang S-J, Vasan RS, Larson MG, Pencina MJ, Hamburg NM, Vita JA, Levy D & Benjamin EJ. (2010). Arterial Stiffness and Cardiovascular Events. *Circulation* **121**, 505-511.

Miyachi M, Kawano H, Sugawara J, Takahashi K, Hayashi K, Yamazaki K, Tabata I & Tanaka H. (2004). Unfavorable Effects of Resistance Training on Central Arterial Compliance: A Randomized Intervention Study. *Circulation* **110**, 2858-2863.

Miyaki A, Maeda S, Yoshizawa M, Misono M, Saito Y, Sasai H, Endo T, Nakata Y, Tanaka K & Ajisaka R. (2009a). Effect of Weight Reduction With Dietary Intervention on Arterial Distensibility and Endothelial Function in Obese Men. *Angiology* **60**, 351-357.

Miyaki A, Maeda S, Yoshizawa M, Misono M, Saito Y, Sasai H, Kim M-K, Nakata Y, Tanaka K & Ajisaka R. (2009b). Effect of Habitual Aerobic Exercise on Body Weight and Arterial Function in Overweight and Obese Men. *The American Journal of Cardiology* **104**, 823-828.

Möbius-Winkler S, Hilberg T, Menzel K, Golla E, Burman A, Schuler G & Adams V. (2009). Time-dependent mobilization of circulating progenitor cells during strenuous exercise in healthy individuals. *Journal of Applied Physiology* **107**, 1943-1950.

Moien-afshari F, Ghosh P, Khazaei M, kieffer TJ, Brownsey RW & Laher I. (2008). Exercise restores endothelial function independently of weight loss or hyperglycaemic status in db/db mice. *Diabetologia* **51**, 1327-1337. doi: 1310.1007/s00125-00008-00996-x. Epub 02008 Apr 00125.

Moore XL, Michell D, Lee S, Skilton MR, Nair R, Dixon JB, Dart AM & Chin-Dusting J. (2013). Increased Carotid Intima-Media Thickness and Reduced Distensibility in Human Class III Obesity: Independent and Differential Influences of Adiposity and Blood Pressure on the Vasculature. *PLoS ONE* **8**, e53972.

Moreau KL, Donato AJ, Seals DR, DeSouza CA & Tanaka H. (2003). Regular exercise, hormone replacement therapy and the age-related decline in carotid arterial compliance in healthy women. *Cardiovascular Research* **57**, 861-868.

Moreau KL, Hildreth KL, Meditz AL, Deane KD & Kohrt WM. (2012). Endothelial Function Is Impaired across the Stages of the Menopause Transition in Healthy Women. *J Clin Endocrinol Metab* **97**, 4692-4700.

Morici G, Zangla D, Santoro A, Pelosi E, Petrucci E, Gioia M, Bonanno A, Profita M, Bellia V, Testa U & Bonsignore MR. (2005). Supramaximal exercise mobilizes hematopoietic progenitors and reticulocytes in athletes. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* **289**, R1496-R1503.

Moriguchi J, Itoh H, Harada S, Takeda K, Hatta T, Nakata T & Sasaki S. (2005). Low Frequency Regular Exercise Improves Flow-Mediated Dilatation of Subjects with Mild Hypertension. *Hypertens Res* **28**, 315-321.

Mosca L, Benjamin EJ, Berra K, Bezanson JL, Dolor RJ, Lloyd-Jones DM, Newby LK, Pina IL, Roger VL, Shaw LJ, Zhao D, Beckie TM, Bushnell C, D'Armiento J, Kris-Etherton PM, Fang J, Ganiats TG, Gomes AS, Gracia CR, Haan CK, Jackson EA, Judelson DR, Kelepouris E, Lavie CJ, Moore A, Nussmeier NA, Ofili E, Oparil S, Ouyang P, Pinn VW, Sherif K, Smith SC, Jr, Sopko G, Chandra-Strobos N, Urbina EM, Vaccarino V & Wenger NK. (2011). Effectiveness-Based Guidelines for the Prevention of Cardiovascular Disease in Women--2011 Update: A Guideline From the American Heart Association. *Circulation* **123**, 1243-1262.

Motohiro M, Yuasa F, Hattori T, Sumimoto T, Takeuchi M, Kaida M, Jikuhara T, Hikosaka M, Sugiura T & Iwasaka T. (2005). Cardiovascular adaptations to exercise training after uncomplicated acute myocardial infarction. *Am J Phys Med Rehabil* **84**, 684-691.

Mullen MJ, Kharbanda RK, Cross J, Donald AE, Taylor M, Vallance P, Deanfield JE & MacAllister RJ. (2001). Heterogenous Nature of Flow-Mediated Dilatation in Human Conduit Arteries In Vivo. *Circulation Research* **88**, 145-151.

Muller-Ehmsen J, Braun D, Schneider T, Pfister R, Worm N, Wielckens K, Scheid C, Frommolt P & Flesch M. (2008). Decreased number of circulating progenitor cells in obesity: beneficial effects of weight reduction. *European Heart Journal* **29**, 1560-1568.

Munakata M, Ito N, Nunokawa T & Yoshinaga K. (2003). Utility of automated brachial ankle pulse wave velocity measurements in hypertensive patients. *American Journal of Hypertension* **16**, 653-657.

Myers J, Gullestad L, Vagelos R, Do D, Bellin D, Ross H & Fowler MB. (1998). Clinical, Hemodynamic, and Cardiopulmonary Exercise Test Determinants of Survival in Patients Referred for Evaluation of Heart Failure. *Annals of Internal Medicine* **129**, 286-293.

Natoli AK, Medley TL, Ahimastos AA, Drew BG, Thearle DJ, Dilley RJ & Kingwell BA. (2005). Sex Steroids Modulate Human Aortic Smooth Muscle Cell Matrix Protein Deposition and Matrix Metalloproteinase Expression. *Hypertension* **46**, 1129-1134.

Neunteufl T, Heher S, Katzenschlager R, Wölfl G, Kostner K, Maurer G & Weidinger F. (2000). Late prognostic value of flow-mediated dilation in the brachial artery of patients with chest pain. *The American Journal of Cardiology* **86**, 207-210.

Neunteufl T, Katzenschlager R, Hassan A, Klaar U, Schwarzacher S, Glogar D, Bauer P & Weidinger F. (1997). Systemic endothelial dysfunction is related to the extent and severity of coronary artery disease. *Atherosclerosis* **129**, 111-118.

Nichols WW & O'Rourke MF. (2005). *McDonald's Blood Flow in Arteries: Theoretical, Experimental and Clinical Principles*. Oxford University Press, London.

Nowak WN, Mika P, Nowobilski R, Kusinska K, Bukowska-Strakova K, Nizankowski R, Jozkowicz A, Szczeklik A & Dulak J. (2012). Exercise training in intermittent claudication: effects on antioxidant genes, inflammatory mediators and proangiogenic progenitor cells. *Thromb Haemost* **108**, 5.

O'Rourke M. (1990). Arterial stiffness, systolic blood pressure, and logical treatment of arterial hypertension. *Hypertension* **15**, 339-347.

O'Rourke MF, Staessen JA, Vlachopoulos C, Duprez D & Plante GéE. (2002). Clinical applications of arterial stiffness; definitions and reference values. *American Journal of Hypertension* **15**, 426-444.

Oemar BS, Tschudi MR, Godoy N, Brovkovich V, Malinski T & Lüscher TF. (1998). Reduced Endothelial Nitric Oxide Synthase Expression and Production in Human Atherosclerosis. *Circulation* **97**, 2494-2498.

Ormerod MG, ed. (2000). *Flow Cytometry: a practical approach*. Oxford University Press, Oxford.

Padilla J, Johnson BD, Newcomer SC, Wilhite DP, Mickleborough TD, Fly AD, Mather KJ & Wallace JP. (2009). Adjusting Flow-Mediated Dilation for Shear Stress Stimulus Allows Demonstration of Endothelial Dysfunction in a Population with Moderate Cardiovascular Risk. *Journal of Vascular Research* **46**, 592-600.

Palmer RM, Ferrige AG & Moncada S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**, 524-526.

Palmer RM & Moncada S. (1989). A novel citrulline-forming enzyme implicated in the formation of nitric oxide by vascular endothelial cells. *Biochem Biophys Res Commun* **158**, 348-352.

Pedersen BK & Hoffman-Goetz L. (2000). Exercise and the Immune System: Regulation, Integration, and Adaptation. *Physiological Reviews* **80**, 1055-1081.

Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MAS & Rafii S. (2000). Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors. *Blood* **95**, 952-958.

Peng X, Haldar S, Deshpande S, Irani K & Kass DA. (2003). Wall Stiffness Suppresses Akt/eNOS and Cytoprotection in Pulse-Perfused Endothelium. *Hypertension* **41**, 378-381.

Perticone F, Ceravolo R, Candigliota M, Ventura G, Iacopino S, Sinopoli F & Mattioli PL. (2001). Obesity and Body Fat Distribution Induce Endothelial Dysfunction by Oxidative Stress. *Diabetes* **50**, 159-165.

Pierce GL, Beske SD, Lawson BR, Southall KL, Benay FJ, Donato AJ & Seals DR. (2008). Weight Loss Alone Improves Conduit and Resistance Artery Endothelial Function in Young and Older Overweight/Obese Adults. *Hypertension* **52**, 72-79.

Poole DC, Hirai DM, Copp SW & Musch TI. (2012). Muscle oxygen transport and utilization in heart failure: implications for exercise (in)tolerance. *American Journal of Physiology - Heart and Circulatory Physiology* **302**, H1050-H1063.

Pyke KE, Dwyer EM & Tschakovsky ME. (2004). Impact of controlling shear rate on flow-mediated dilation responses in the brachial artery of humans. *J Appl Physiol* **97**, 499-508.

Pyke KE & Tschakovsky ME. (2005). The relationship between shear stress and flow-mediated dilatation: implications for the assessment of endothelial function. *The Journal of Physiology* **568**, 357-369.

Pyke KE & Tschakovsky ME. (2007). Peak vs. total reactive hyperemia: which determines the magnitude of flow-mediated dilation? *J Appl Physiol* **102**, 1510-1519.

Radomski MW, Palmer RM & Moncada S. (1990). An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc Natl Acad Sci USA* **87**, 5193-5197.

Rakobowchuk M, Harris E, Taylor A, Baliga V, Cubbon RM, Rossiter HB & Birch KM. (2012). Heavy and moderate interval exercise training alters low-flow-mediated constriction but does not increase circulating progenitor cells in healthy humans. *Experimental Physiology* **97**, 375-385.

Rakobowchuk M, Harris E, Taylor A, Cubbon R & Birch K. (2013). Moderate and heavy metabolic stress interval training improve arterial stiffness and heart rate dynamics in humans. *European Journal of Applied Physiology* **113**, 839-849.

Rakobowchuk M, McGowan CL, de Groot PC, Bruinsma D, Hartman JW, Phillips SM & MacDonald MJ. (2005). Effect of whole body resistance training on arterial compliance in young men. *Experimental Physiology* **90**, 645-651.

Rakobowchuk M, Tanguay S, Burgomaster KA, Howarth KR, Gibala MJ & MacDonald MJ. (2008). Sprint interval and traditional endurance training induce similar improvements in peripheral arterial stiffness and flow-mediated dilation in healthy humans. *Am J Physiol Regul Integr Comp Physiol* **295**, R236-242.

Rehman J, Li J, Orschell CM & March KL. (2003). Peripheral Blood "Endothelial Progenitor Cells" Are Derived From Monocyte/Macrophages and Secrete Angiogenic Growth Factors. *Circulation* **107**, 1164-1169.

Rehman J, Li J, Parvathaneni L, Karlsson G, Panchal VR, Temm CJ, Mahenthiran J & March KL. (2004). Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. *Journal of the American College of Cardiology* **43**, 2314-2318.

Rivard A, Fabre J-E, Silver M, Chen D, Murohara T, Kearney M, Magner M, Asahara T & Isner JM. (1999). Age-Dependent Impairment of Angiogenesis. *Circulation* **99**, 111-120.

Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, Carnethon MR, Dai S, de Simone G, Ford ES, Fox CS, Fullerton HJ, Gillespie C, Greenlund KJ, Hailpern SM, Heit JA, Ho PM, Howard VJ, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Makuc DM, Marcus GM, Marelli A, Matchar DB, McDermott MM, Meigs JB, Moy CS, Mozaffarian D, Mussolino ME, Nichol G, Paynter NP, Rosamond WD, Sorlie PD, Stafford RS, Turan TN, Turner MB, Wong ND, Wylie-Rosett J, on behalf of the American Heart Association Statistics Committee Stroke Statistics Subcommittee & On behalf of the American Heart Association Heart Disease Stroke Statistics Writing Group. (2011). Heart Disease and Stroke Statistics--2011 Update: A Report From the American Heart Association. *Circulation* **123**, e18-209.

Rognmo O, Bjornstad TH, Kahrs C, Tjonna AE, Bye A, Haram PM, Stolen T, Slordahl SA & Wisloff U. (2008). Endothelial Function in Highly Endurance-Trained Men: Effects of Acute Exercise. *Journal of Strength & Conditioning Research March* **22**, 535-542.

Rohde E, Bartmann C, Schallmoser K, Reinisch A, Lanzer G, Linkesch W, Guelly C & Strunk D. (2007). Immune Cells Mimic the Morphology of Endothelial Progenitor Colonies In Vitro. *STEM CELLS* **25**, 1746-1752.

Rohde E, Malischnik C, Thaler D, Maierhofer T, Linkesch W, Lanzer G, Guelly C & Strunk D. (2006). Blood Monocytes Mimic Endothelial Progenitor Cells. *STEM CELLS* **24**, 357-367.

Ross R. (1999). Atherosclerosis — An Inflammatory Disease. *New England Journal of Medicine* **340**, 115-126.

Rossiter HB. (2011). Exercise: Kinetic Considerations for Gas Exchange. In *Comprehensive Physiology*, vol. 1, pp. 203-244.

Rossiter HB, Kowalchuk JM & Whipp BJ. (2006). A test to establish maximum O2 uptake despite no plateau in the O2 uptake response to ramp incremental exercise. *Journal of Applied Physiology* **100**, 764-770.

Rousseau A, Ayoubi F, Deveaux C, Charbit B, Delmau C, Christin-Maitre S, Jaillon P, Uzan G & Simon T. (2010). Impact of age and gender interaction on circulating endothelial progenitor cells in healthy subjects. *Fertility and Sterility* **93**, 843-846.

Rubanyi GM. (1993). The role of endothelium in cardiovascular homeostasis and diseases. *J Cardiovasc Pharmacol* **22 Suppl 4**, S1-14.

Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS & Sessa WC. (1998). Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest* **101**, 731-736.

Sandri M, Adams V, Gielen S, Linke A, Lenk K, Kränkel N, Lenz D, Erbs S, Scheinert D, Mohr FW, Schuler G & Hambrecht R. (2005). Effects of Exercise and Ischemia on Mobilization and Functional Activation of Blood-Derived Progenitor Cells in Patients With Ischemic Syndromes. *Circulation* **111**, 3391-3399.

Sarto P, Balducci E, Balconi G, Fiordaliso F, Merlo L, Tuzzato G, Pappagallo GL, Frigato N, Zanocco A, Forestieri C, Azzarello G, Mazzucco A, Valenti MT, Alborino F, Noventa D, Vinante O, Pascotto P, Sartore S, Dejana E & Latini R. (2007). Effects of Exercise Training on Endothelial Progenitor Cells in Patients With Chronic Heart Failure. *J Card Fail* **13**, 701-708.

Scharhag-Rosenberger F, Meyer T, Gäßler N, Faude O & Kindermann W. (2010). Exercise at given percentages of VO2max: Heterogeneous metabolic responses between individuals. *Journal of Science and Medicine in Sport* **13**, 74-79.

Scheubel RJ, Kahrstedt S, Weber H, Holtz J, Friedrich I, Borgermann J, Silber R-E & Simm A. (2006). Depression of progenitor cell function by advanced glycation endproducts (AGEs): Potential relevance for impaired angiogenesis in advanced age and diabetes. *Exp Gerontol* **41**, 540-548.

Scheubel RJ, Zorn H, Silber R-E, Kuss O, Morawietz H, Holtz J & Simm A. (2003). Age-dependent depression in circulating endothelial progenitor cells inpatients undergoing coronary artery bypass grafting. *Journal of the American College of Cardiology* **42**, 2073-2080.

Schjerve IE, Tyldum GA, Tjønna AE, Stølen T, Loennechen JP, Hansen HEM, Haram PM, Heinrich G, Bye A, Najjar SM, Smith GL, Slørdahl SA, Kemi OJ & Wisløff U. (2008). Both aerobic endurance and strength training programmes improve cardiovascular health in obese adults. *Clinical Science* **115**, 283-293.

Schlager O, Giurgea A, Schuhfried O, Seidinger D, Hammer A, Gröger M, Fialka-Moser V, Gschwandtner M, Koppensteiner R & Steiner S. (2011). Exercise training increases endothelial progenitor cells and decreases asymmetric dimethylarginine in peripheral arterial disease: A randomized controlled trial. *Atherosclerosis* **217**, 240-248.

Schmidt-Lucke C, Fichtlscherer S, Aicher A, Tschöpe C, Schultheiss H-P, Zeiher AM & Dimmeler S. (2010). Quantification of Circulating Endothelial Progenitor Cells Using the Modified ISHAGE Protocol. *PLoS ONE* **5**, e13790.

Schmidt-Lucke C, Rössig L, Fichtlscherer S, Vasa M, Britten M, Kämper U, Dimmeler S & Zeiher AM. (2005). Reduced Number of Circulating Endothelial Progenitor Cells Predicts Future Cardiovascular Events: Proof of Concept for the Clinical Importance of Endogenous Vascular Repair. *Circulation* **111**, 2981-2987.

Scuteri A, Cacciafesta M, Di Bernardo MG, De Propris AM, Recchi D, Celli V, Cicconetti P & Marigliano V. (1995). Pulsatile versus steady-state component of blood pressure in elderly females: an independent risk factor for cardiovascular disease? *J Hypertens* **13**, 185-191.

Seals DR, Tanaka H, Clevenger CM, Monahan KD, Reiling MJ, Hiatt WR, Davy KP & DeSouza CA. (2001). Blood pressure reductions with exercise and sodium restriction in postmenopausal women with elevated systolic pressure: role of arterial stiffness. *Journal of the American College of Cardiology* **38**, 506-513.

Seeger FH, Haendeler J, Walter DH, Rochwalsky U, Reinhold J, Urbich C, Rössig L, Corbaz A, Chvatchko Y, Zeiher AM & Dimmeler S. (2005). p38 Mitogen-Activated Protein Kinase Downregulates Endothelial Progenitor Cells. *Circulation* **111**, 1184-1191.

Selzer RH, Mack WJ, Lee PL, Kwong-Fu H & Hodis HN. (2001). Improved common carotid elasticity and intima-media thickness measurements from computer analysis of sequential ultrasound frames. *Atherosclerosis* **154**, 185-193.

Sessa WC, Pritchard K, Seyedi N, Wang J & Hintze TH. (1994). Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circulation Research* **74**, 349-353.

Shaffer RG, Greene S, Arshi A, Supple G, Bantly A, Moores JS, Parmacek MS & Mohler ER. (2006). Effect of acute exercise on endothelial progenitor cells in patients with peripheral arterial disease. *Vascular Medicine* **11**, 219-226.

Shi Q, Rafii S, Wu MH-D, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MAS, Storb RF & Hammond WP. (1998). Evidence for Circulating Bone Marrow-Derived Endothelial Cells. *Blood* **92**, 362-367.

Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K-i, Shimada T, Oike Y & Imaizumi T. (2001). Mobilization of Endothelial Progenitor Cells in Patients With Acute Myocardial Infarction. *Circulation* **103**, 2776-2779.

Silva CAd, Ribeiro JP, Canto JCAU, Silva REd, Silva Junior GB, Botura E & Malschitzky MAR. (2012). High-intensity aerobic training improves endothelium-

dependent vasodilation in patients with metabolic syndrome and type 2 diabetes mellitus. *Diabetes Research and Clinical Practice* **95**, 237-245.

Simmons GH, Padilla J, Young CN, Wong BJ, Lang JA, Davis MJ, Laughlin MH & Fadel PJ. (2011). Increased brachial artery retrograde shear rate at exercise onset is abolished during prolonged cycling: role of thermoregulatory vasodilation. *Journal of Applied Physiology* **110**, 389-397.

Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW & Liao JK. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* **407**, 538-541.

Simons PCG, Algra A, Bots ML, Grobbee DE, van der Graaf Y & Group ftSS. (1999). Common Carotid Intima-Media Thickness and Arterial Stiffness. *Circulation* **100**, 951-957.

Singh R, Barden A, Mori T & Beilin L. (2001). Advanced glycation end-products: a review. *Diabetologia* **44**, 129-146.

Smulyan H, Asmar RG, Rudnicki A, London GM & Safar ME. (2001). Comparative effects of aging in men and women on the properties of the arterial tree. *Journal of the American College of Cardiology* **37**, 1374-1380.

Sonnenschein K, Horvath T, Mueller M, Markowski A, Siegmund T, Jacob C, Drexler H & Landmesser U. (2011). Exercise training improves in vivo endothelial repair capacity of early endothelial progenitor cells in subjects with metabolic syndrome. *Eur J Cardiovasc Prev Rehabil* **18**, 406-414. Epub 2011 Feb 2011.

Sorensen KE, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge DJ & Deanfield JE. (1994). Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. *J Clin Invest* **93**, 50-55.

Sorescu D, Weiss D, Lassègue B, Clempus RE, Szöcs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor WR & Griendling KK. (2002). Superoxide Production and Expression of Nox Family Proteins in Human Atherosclerosis. *Circulation* **105**, 1429-1435.

Soucy KG, Ryoo S, Benjo A, Lim HK, Gupta G, Sohi JS, Elser J, Aon MA, Nyhan D, Shoukas AA & Berkowitz DE. (2006). Impaired shear stress-induced nitric oxide production through decreased NOS phosphorylation contributes to age-related vascular stiffness. *Journal of Applied Physiology* **101**, 1751-1759.

Sprung VS, Cuthbertson DJ, Pugh CJA, Aziz N, Kemp GJ, Daousi C, Green DJ, Tim Cable N & Jones H. (2013). Exercise Training in PCOS Enhances FMD in the Absence of Changes in Fatness. *Medicine & Science in Sports & Exercise* **Publish Ahead of Print**, 10.1249/MSS.1240b1013e31829ba31829a31821.

Stamler J, Rose G, Stamler R, Elliott P, Dyer A & Marmot M. (1989). INTERSALT study findings. Public health and medical care implications. *Hypertension* **14**, 570-577.

Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, Najjar SS, Rembold CM & Post WS. (2008). Use of Carotid Ultrasound to Identify Subclinical Vascular Disease and Evaluate Cardiovascular Disease Risk: A Consensus Statement from the American Society of Echocardiography Carotid Intima-

Media Thickness Task Force Endorsed by the Society for Vascular Medicine. *Journal of the American Society of Echocardiography* **21**, 93-111.

Steinberg HO, Paradisi G, Cronin J, Crowde K, Hempfling A, Hook G & Baron AD. (2000). Type II Diabetes Abrogates Sex Differences in Endothelial Function in Premenopausal Women. *Circulation* **101**, 2040-2046.

Steiner S, Niessner A, Ziegler S, Richter B, Seidinger D, Pleiner J, Penka M, Wolzt M, Huber K, Wojta J, Minar E & Kopp CW. (2005). Endurance training increases the number of endothelial progenitor cells in patients with cardiovascular risk and coronary artery disease. *Atherosclerosis* **181**, 305-310.

Stensvold D, Tjønna AE, Skaug E-A, Aspenes S, Stølen T, Wisløff U & Slørdahl SA. (2010). Strength training versus aerobic interval training to modify risk factors of metabolic syndrome. *Journal of Applied Physiology* **108**, 804-810.

Strauer BE, Brehm M, Zeus T, Köstering M, Hernandez A, Sorg RV, Kögler G & Wernet P. (2002). Repair of Infarcted Myocardium by Autologous Intracoronary Mononuclear Bone Marrow Cell Transplantation in Humans. *Circulation* **106**, 1913-1918.

Strehlow K, Werner N, Berweiler J, Link A, Dirnagl U, Priller J, Laufs K, Ghaeni L, Milosevic M, Bohm M & Nickenig G. (2003). Estrogen Increases Bone Marrow-Derived Endothelial Progenitor Cell Production and Diminishes Neointima Formation. *Circulation* **107**, 3059-3065.

Sugawara J, Komine H, Hayashi K, Yoshizawa M, Yokoi T, Otsuki T, Shimojo N, Miyauchi T, Maeda S & Tanaka H. (2007). Effect of Systemic Nitric Oxide Synthase Inhibition on Arterial Stiffness in Humans. *Hypertens Res* **30**, 411-415.

Sugawara J, Otsuki T, Tanabe T, Hayashi K, Maeda S & Matsuda M. (2006). Physical Activity Duration, Intensity, and Arterial Stiffening in Postmenopausal Women[ast]. *Am J Hypertens* **19**, 1032-1036.

Swift DL, Earnest CP, Blair SN & Church TS. (2012). The effect of different doses of aerobic exercise training on endothelial function in postmenopausal women with elevated blood pressure: results from the DREW study. *British Journal of Sports Medicine* **46**, 753-758.

Taddei S, Virdis A, Ghiadoni L, Magagna A & Salvetti A. (1998). Vitamin C Improves Endothelium-Dependent Vasodilation by Restoring Nitric Oxide Activity in Essential Hypertension. *Circulation* **97**, 2222-2229.

Taddei S, Virdis A, Ghiadoni L, Mattei P, Sudano I, Bernini G, Pinto S & Salvetti A. (1996). Menopause Is Associated With Endothelial Dysfunction in Women. *Hypertension* **28**, 576-582.

Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A & Salvetti A. (2001). Age-Related Reduction of NO Availability and Oxidative Stress in Humans. *Hypertension* **38**, 274-279.

Taddei S, Virdis A, Mattei P, Ghiadoni L, Gennari A, Fasolo CB, Sudano I & Salvetti A. (1995). Aging and Endothelial Function in Normotensive Subjects and Patients With Essential Hypertension. *Circulation* **91**, 1981-1987.

Takase B, Uehata A, Akima T, Nagai T, Nishioka T, Hamabe A, Satomura K, Ohsuzu F & Kurita A. (1998). Endothelium-dependent flow-mediated vasodilation in coronary and brachial arteries in suspected coronary artery disease. *The American Journal of Cardiology* **82**, 1535-1539.

Tanaka H, DeSouza CA & Seals DR. (1998). Absence of Age-Related Increase in Central Arterial Stiffness in Physically Active Women. *Arteriosclerosis, Thrombosis, and Vascular Biology* **18**, 127-132.

Tanaka H, Dinenno FA, Monahan KD, Clevenger CM, DeSouza CA & Seals DR. (2000). Aging, Habitual Exercise, and Dynamic Arterial Compliance. *Circulation* **102**, 1270-1275.

Tanaka H, Seals DR, Monahan KD, Clevenger CM, DeSouza CA & Dinenno FA. (2002). Regular aerobic exercise and the age-related increase in carotid artery intima-media thickness in healthy men. *Journal of Applied Physiology* **92**, 1458-1464.

Tao J, Jin Y-F, Yang Z, Wang L-C, Gao X-r, Lei L & Ma H. (2004). Reduced arterial elasticity is associated with endothelial dysfunction in persons of advancing age: Comparative study of noninvasive pulse wave analysis and laser doppler blood flow measurement. *American Journal of Hypertension* **17**, 654-659.

Tao J, Wang Y, Yang Z, Tu C, Xu MG & Wang JM. (2006). Circulating endothelial progenitor cell deficiency contributes to impaired arterial elasticity in persons of advancing age. *J Hum Hypertens* **20**, 490-495.

Tare M, Parkington HC, Coleman HA, Neild TO & Dusting GJ. (1990). Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature* **346**, 69-71.

Taylor C, Cheng C, Espinosa L, Tang B, Parker D & Herfkens R. (2002). In Vivo Quantification of Blood Flow and Wall Shear Stress in the Human Abdominal Aorta During Lower Limb Exercise. *Annals of Biomedical Engineering* **30**, 402-408.

Tepper OM, Capla JM, Galiano RD, Ceradini DJ, Callaghan MJ, Kleinman ME & Gurtner GC. (2005). Adult vasculogenesis occurs through in situ recruitment, proliferation, and tubulization of circulating bone marrow–derived cells. *Blood* **105**, 1068-1077.

Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP & Gurtner GC. (2002). Human Endothelial Progenitor Cells From Type II Diabetics Exhibit Impaired Proliferation, Adhesion, and Incorporation Into Vascular Structures. *Circulation* **106**, 2781-2786.

Thacher T, Gambillara V, da Silva RF, Silacci P & Stergiopulos N. (2010). Reduced cyclic stretch, endothelial dysfunction, and oxidative stress: an ex vivo model. *Cardiovascular Pathology* **19**, Jul-Aug.

Thijssen DH, Dawson EA, Black MA, Hopman MT, Cable NT & Green DJ. (2009a). Brachial artery blood flow responses to different modalities of lower limb exercise. *Med Sci Sports Exerc* **41**, 1072-1079.

Thijssen DHJ, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, Parker B, Widlansky ME, Tschakovsky ME & Green DJ. (2011a). Assessment of flow-mediated dilation in humans: a methodological and physiological guideline.

American Journal of Physiology - Heart and Circulatory Physiology **300**, H2-H12.

Thijssen DHJ, Bullens LM, van Bemmel MM, Dawson EA, Hopkins N, Tinken TM, Black MA, Hopman MTE, Cable NT & Green DJ. (2009b). Does arterial shear explain the magnitude of flow-mediated dilation?: a comparison between young and older humans. *Am J Physiol-Heart C* **296**, H57-H64.

Thijssen DHJ, Dawson EA, Tinken TM, Cable NT & Green DJ. (2009c). Retrograde Flow and Shear Rate Acutely Impair Endothelial Function in Humans. *Hypertension* **53**, 986-992.

Thijssen DHJ, Rowley N, Padilla J, Simmons GH, Laughlin MH, Whyte G, Cable NT & Green DJ. (2011b). Relationship between upper and lower limb conduit artery vasodilator function in humans. *Journal of Applied Physiology* **111**, 244-250.

Thijssen DHJ, Vos JB, Verseyden C, Van Zonneveld AJ, Smits P, Sweep FCGJ, Hopman MTE & De Boer HC. (2006). Haematopoietic stem cells and endothelial progenitor cells in healthy men: effect of aging and training. *Aging Cell* **5**, 495-503.

Thorell D, Borjesson M, Larsson P, Ulfhammer E, Karlsson L & DuttaRoy S. (2009). Strenuous exercise increases late outgrowth endothelial cells in healthy subjects. *European Journal of Applied Physiology* **107**, 481-488.

Thum T, Fraccarollo D, Schultheiss M, Froese S, Galuppo P, Widder JD, Tsikas D, Ertl G & Bauersachs J. (2007). Endothelial Nitric Oxide Synthase Uncoupling Impairs Endothelial Progenitor Cell Mobilization and Function in Diabetes. *Diabetes* **56**, 666-674.

Timmermans F, Van Hauwermeiren F, De Smedt M, Raedt R, Plasschaert F, De Buyzere ML, Gillebert TC, Plum J & Vandekerckhove B. (2007). Endothelial Outgrowth Cells Are Not Derived From CD133+ Cells or CD45+ Hematopoietic Precursors. *Arterioscler Thromb Vasc Biol* **27**, 1572-1579.

Tinken TM, Thijssen DHJ, Black MA, Cable NT & Green DJ. (2008). Time course of change in vasodilator function and capacity in response to exercise training in humans. *The Journal of Physiology* **586**, 5003-5012.

Tinken TM, Thijssen DHJ, Hopkins N, Dawson EA, Cable NT & Green DJ. (2010). Shear Stress Mediates Endothelial Adaptations to Exercise Training in Humans. *Hypertension* **55**, 312-318.

Tjønna AE, Lee SJ, Rognmo Ø, Stølen TO, Bye A, Haram PM, Loennechen JP, Al-Share QY, Skogvoll E, Slørdahl SA, Kemi OJ, Najjar SM & Wisløff U. (2008). Aerobic Interval Training Versus Continuous Moderate Exercise as a Treatment for the Metabolic Syndrome. *Circulation* **118**, 346-354.

Tobler K, Freudenthaler A, Baumgartner-Parzer SM, Wolzt M, Ludvik B, Nansalmaa E, Nowotny PJ, Seidinger D, Steiner S, Luger A & Artwohl M. (2010). Reduction of both number and proliferative activity of human endothelial progenitor cells in obesity. *Int J Obes (Lond)* **34**, 687-700. doi: 610.1038/ijo.2009.1280. Epub 2010 Jan 1012.

Tordi N, Mourot L, Colin E & Regnard J. (2010). Intermittent versus constant aerobic exercise: effects on arterial stiffness. *European Journal of Applied Physiology* **108**, 801-809.

Turner AP, Cathcart AJ, Parker ME, Butterworth C, Wilson J & Ward SA. (2006). Oxygen Uptake and Muscle Desaturation Kinetics during Intermittent Cycling. *Medicine & Science in Sports & Exercise* **38**, 492-503

Urbich C & Dimmeler S. (2004). Endothelial Progenitor Cells. *Circulation Research* **95**, 343-353.

Uribarri J, Cai W, Peppa M, Goodman S, Ferrucci L, Striker G & Vlassara H. (2007). Circulating Glycotoxins and Dietary Advanced Glycation Endproducts: Two Links to Inflammatory Response, Oxidative Stress, and Aging. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **62**, 427-433.

Vaccarino V, Parsons L, Peterson ED, Rogers WJ, Kiefe CI & Canto J. (2009). Sex differences in mortality after acute myocardial infarction: Changes from 1994 to 2006. *Archives of Internal Medicine* **169**, 1767-1774.

Vaitkevicius PV, Fleg JL, Engel JH, O'Connor FC, Wright JG, Lakatta LE, Yin FC & Lakatta EG. (1993). Effects of age and aerobic capacity on arterial stiffness in healthy adults. *Circulation* **88**, 1456-1462.

Vallance P & Leiper J. (2004). Cardiovascular Biology of the Asymmetric Dimethylarginine:Dimethylarginine Dimethylaminohydrolase Pathway. *Arterioscler Thromb Vasc Biol* **24**, 1023-1030.

Van Craenenbroeck EM, Beckers PJ, Possemiers NM, Wuyts K, Frederix G, Hoymans VY, Wuyts F, Paelinck BP, Vrints CJ & Conraads VM. (2010). Exercise acutely reverses dysfunction of circulating angiogenic cells in chronic heart failure. *European Heart Journal* **31**, 1924-1934.

Van Craenenbroeck EMF, Vrints CJ, Haine SE, Vermeulen K, Goovaerts I, Van Tendeloo VFI, Hoymans VY & Conraads VMA. (2008). A maximal exercise bout increases the number of circulating CD34+/KDR+ endothelial progenitor cells in healthy subjects. Relation with lipid profile. *Journal of applied physiology (Bethesda, Md : 1985)* **104**, 1006-1013.

van der Heijden-Spek JJ, Staessen JA, Fagard RH, Hoeks AP, Boudier HAS & Van Bortel LM. (2000). Effect of Age on Brachial Artery Wall Properties Differs From the Aorta and Is Gender Dependent: A Population Study. *Hypertension* **35**, 637-642.

Van Popele NM, Grobbee DE, Bots ML, Asmar R, Topouchian J, Reneman RS, Hoeks APG, van der Kuip DAM, Hofman A & Witteman JCM. (2001). Association Between Arterial Stiffness and Atherosclerosis : The Rotterdam Study. *Stroke* **32**, 454-460.

Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM & Dimmeler S. (2001). Number and Migratory Activity of Circulating Endothelial Progenitor Cells Inversely Correlate With Risk Factors for Coronary Artery Disease. *Circ Res* **89**, e1-7.

Verma S, Kuliszewski MA, Li S-H, Szmitko PE, Zucco L, Wang C-H, Badiwala MV, Mickle DAG, Weisel RD, Fedak PWM, Stewart DJ & Kutryk MJB. (2004). C-Reactive Protein Attenuates Endothelial Progenitor Cell Survival, Differentiation, and Function: Further Evidence of a Mechanistic Link Between C-Reactive Protein and Cardiovascular Disease. *Circulation* **109**, 2058-2067.

Virdis A, Ghiadoni L, Pinto S, Lombardo M, Petraglia F, Gennazzani A, Buralli S, Taddei S & Salvetti A. (2000). Mechanisms Responsible for Endothelial Dysfunction Associated With Acute Estrogen Deprivation in Normotensive Women. *Circulation* **101**, 2258-2263.

Vogel T, Leprêtre PM, Brechat PH, Lonsdorfer-Wolf E, Kaltenbach G, Lonsdorfer J & Benetos A. (2013). Effect of a short-term intermittent exercise-training programme on the pulse wave velocity and arterial pressure: a prospective study among 71 healthy older subjects. *Int J Clin Pract* **67**, 420-426.

Wada T, Kodaira K, Fujishiro K, Maie K, Tsukiyama E, Fukumoto T, Uchida T & Yamazaki S. (1994). Correlation of ultrasound-measured common carotid artery stiffness with pathological findings. *Arteriosclerosis, Thrombosis, and Vascular Biology* **14**, 479-482.

Waldman SA & Murad F. (1988). Biochemical mechanisms underlying vascular smooth muscle relaxation: the guanylate cyclase-cyclic GMP system. *J Cardiovasc Pharmacol* **12**, S115-118.

Walsh BW, Schiff I, Rosner B, Greenberg L, Ravnikar V & Sacks FM. (1991). Effects of Postmenopausal Estrogen Replacement on the Concentrations and Metabolism of Plasma Lipoproteins. *New England Journal of Medicine* **325**, 1196-1204.

Walsh JH, Bilsborough W, Maiorana A, Best M, O'Driscoll GJ, Taylor RR & Green DJ. (2003). Exercise training improves conduit vessel function in patients with coronary artery disease. *Journal of Applied Physiology* **95**, 20-25.

Walter DH, Haendeler J, Reinhold J, Rochwalsky U, Seeger F, Honold J, Hoffmann J, Urbich C, Lehmann R, Arenzana-Seisdesdos F, Aicher A, Heeschen C, Fichtlscherer S, Zeiher AM & Dimmeler S. (2005). Impaired CXCR4 Signaling Contributes to the Reduced Neovascularization Capacity of Endothelial Progenitor Cells From Patients With Coronary Artery Disease. *Circulation Research* **97**, 1142-1151.

Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, Nishimura H, Losordo DW, Asahara T & Isner JM. (2002). Statin Therapy Accelerates Reendothelialization: A Novel Effect Involving Mobilization and Incorporation of Bone Marrow-Derived Endothelial Progenitor Cells. *Circulation* **105**, 3017-3024.

Wang Y, Wang S, Wier WG, Zhang Q, Jiang H, Li Q, Chen S, Tian Z, Li Y, Yu X, Zhao M, Liu J, Yang J, Zhang J & Zang W. (2010). Exercise improves the dilatation function of mesenteric arteries in postmyocardial infarction rats via a PI3K/Akt/eNOS pathway-mediated mechanism. *American Journal of Physiology - Heart and Circulatory Physiology* **299**, H2097-H2106.

Wang YC, McPherson K, Marsh T, Gortmaker SL & Brown M. (2011). Health and economic burden of the projected obesity trends in the USA and the UK. *The Lancet* **378**, 815-825.

Wenger NK. (2012). Women and Coronary Heart Disease: A Century After Herrick. *Circulation* **126**, 604-611.

Werner C, Fürster T, Widmann T, Pöss J, Roggia C, Hanhoun M, Scharhag J, Büchner N, Meyer T, Kindermann W, Haendeler J, Böhm M & Laufs U. (2009). Physical Exercise Prevents Cellular Senescence in Circulating Leukocytes and in the Vessel Wall. *Circulation* **120**, 2438-2447.

Werner N, Junk S, Laufs U, Link A, Walenta K, Böhm M & Nickenig G. (2003). Intravenous Transfusion of Endothelial Progenitor Cells Reduces Neointima Formation After Vascular Injury. *Circulation Research* **93**, e17-e24.

Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Böhm M & Nickenig G. (2005). Circulating Endothelial Progenitor Cells and Cardiovascular Outcomes. *New England Journal of Medicine* **353**, 999-1007.

Westendorp ICD, Bots ML, Grobbee DE, Reneman RS, Hoeks APG, Van Popele NM, Hofman A & Witteman JCM. (1999). Menopausal Status and Distensibility of the Common Carotid Artery. *Arteriosclerosis, Thrombosis, and Vascular Biology* **19**, 713-717.

Whipp BJ, Ward SA & Wasserman K. (1986). Respiratory markers of the anaerobic threshold. *Adv Cardiol* **35**, 47-64.

Whyte LJ, Ferguson C, Wilson J, Scott RA & Gill JMR. (2013). Effects of single bout of very high-intensity exercise on metabolic health biomarkers in overweight/obese sedentary men. *Metabolism* **62**, 212-219.

Whyte LJ, Gill JMR & Cathcart AJ. (2010). Effect of 2 weeks of sprint interval training on health-related outcomes in sedentary overweight/obese men. *Metabolism: clinical and experimental* **59**, 1421-1428.

Wildman RP, Mackey RH, Bostom A, Thompson T & Sutton-Tyrrell K. (2003). Measures of Obesity Are Associated With Vascular Stiffness in Young and Older Adults. *Hypertension* **42**, 468-473.

Wilkinson IB, Fuchs SA, Jansen IM, Spratt JC, Murray GD, Cockcroft JR & Webb DJ. (1998). Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis. *Journal of Hypertension* **16**, 2079-2084.

Wilkinson IB, MacCallum H, Cockcroft JR & Webb DJ. (2002). Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. *British Journal of Clinical Pharmacology* **53**, 189-192.

Williamson K, Stringer SE & Alexander MY. (2012). Endothelial progenitor cells enter the aging arena. *Front Physiol* **3**, 30. Epub 2012 Feb 2020.

Williamson KA, Hamilton A, Reynolds JA, Sipos P, Crocker I, Stringer SE & Alexander YM. (2013). Age-related impairment of endothelial progenitor cell migration correlates with structural alterations of heparan sulfate proteoglycans. *Aging Cell* **12**, 139-147.

Wisloff U, Stoylen A, Loennechen JP, Bruvold M, Rognmo O, Haram PM, Tjonna AE, Helgerud J, Slordahl SA, Lee SJ, Videm V, Bye A, Smith GL, Najjar SM, Ellingsen O & Skjaerpe T. (2007). Superior Cardiovascular Effect of Aerobic Interval Training Versus Moderate Continuous Training in Heart Failure Patients: A Randomized Study. *Circulation* **115**, 3086-3094.

Woods JA, Vieira VJ & Keylock KT. (2009). Exercise, Inflammation, and Innate Immunity. *Immunology and Allergy Clinics of North America* **29**, 381-393.

World Health Organisation. (2009). Global health risks. Mortality and burden of disease attributable to selected major risks. World Health Organisation, Geneva.

World Health Organisation. (2011). World Health organisation: Global Atlas on Cardiovascular Disease Prevention and Control. World Health Organisation, Geneva.

Woywodt A, Bahlmann FH, De Groot K, Haller H & Haubitz M. (2002). Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer. *Nephrol Dial Transplant* **17**, 1728-1730.

Xia W-H, Li J, Su C, Yang Z, Chen L, Wu F, Zhang Y-Y, Yu B-B, Qiu Y-X, Wang S-M & Tao J. (2012). Physical exercise attenuates age-associated reduction in endothelium-reparative capacity of endothelial progenitor cells by increasing CXCR4/JAK-2 signaling in healthy men. *Aging Cell* **11**, 111-119.

Yamashina A, Tomiyama H, Arai T, Hirose K, Koji Y, Hirayama Y, Yamamoto Y & Hori S. (2003). Brachial-ankle pulse wave velocity as a marker of atherosclerotic vascular damage and cardiovascular risk. *Hypertension research : official journal of the Japanese Society of Hypertension* **26**, 615-622.

Yamashina A, Tomiyama H, Takeda K, Tsuda H, Arai T, Hirose K, Koji Y, Hori S & Yamamoto Y. (2002). Validity, Reproducibility, and Clinical Significance of Noninvasive Brachial-Ankle Pulse Wave Velocity Measurement. *Hypertension Research* **25**, 359-364.

Yang SJ, Hong HC, Choi HY, Yoo HJ, Cho GJ, Hwang TG, Baik SH, Choi DS, Kim SM & Choi KM. (2011). Effects of a three-month combined exercise programme on fibroblast growth factor 21 and fetuin-A levels and arterial stiffness in obese women. *Clin Endocrinol* **75**, 464-469.

Yang Z, Xia W-H, Su C, Wu F, Zhang Y-Y, Xu S-Y, Liu X, Zhang X-Y, Ou Z-J, Lai G-H, Liao X-X, Jin Y-F & Tao J. (2013). Regular exercise-induced increased number and activity of circulating endothelial progenitor cells attenuates age-related decline in arterial elasticity in healthy men. *International Journal of Cardiology* **165**, 247-254.

Yasmin, McEniery CM, Wallace S, Dakham Z, Pusalkar P, Maki-Petaja K, Ashby MJ, Cockcroft JR & Wilkinson IB. (2005). Matrix Metalloproteinase-9 (MMP-9), MMP-2, and Serum Elastase Activity Are Associated With Systolic Hypertension and Arterial Stiffness. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25**, 372-378.

Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT & Ingram DA. (2007). Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* **109**, 1801-1809.

Zampetaki A, Kirton JP & Xu Q. (2008). Vascular repair by endothelial progenitor cells. *Cardiovascular Research* **78**, 413-421.

Zaros PR, Pires CEMR, Bacci M, Jr., Moraes C & Zanesco A. (2009). Effect of 6-months of physical exercise on the nitrate/nitrite levels in hypertensive postmenopausal women. *BMC Womens Health* **9**, 17.

Zebekakis PE, Nawrot T, Thijs L, Balkestein EJ, van der Heijden-Spek J, Van Bortel LM, Struijker-Boudier HA, Safar ME & Staessen JA. (2005). Obesity is associated with increased arterial stiffness from adolescence until old age. *Journal of Hypertension* **23**, 1839-1846.

Zentilin L, Tafuro S, Zacchigna S, Arsic N, Pattarini L, Sinigaglia M & Giacca M. (2006). Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. *Blood* **107**, 3546-3554.

Zhang Y, Lee TS, Kolb EM, Sun K, Lu X, Sladek FM, Kassab GS, Garland T, Jr. & Shyy JY. (2006). AMP-activated protein kinase is involved in endothelial NO synthase activation in response to shear stress. *Arteriosclerosis, Thrombosis & Vascular Biology* **26**, 1281-1287.

Zheng H, Fu G, Dai T & Huang H. (2007). Migration of Endothelial Progenitor Cells Mediated by Stromal Cell-Derived Factor-1[alpha]/CXCR4 via PI3K/Akt/eNOS Signal Transduction Pathway. *J Cardiovasc Pharmacol* **50**, 274-280 210.1097/FJC.1090b1013e318093ec318098f.

Zheng H, Shen C-J, Qiu F-Y, Zhao Y-B & Fu G-S. (2010). Stromal cell-derived factor 1α reduces senescence of endothelial progenitor subpopulation in lectin-binding and DiLDL-uptaking cell through telomerase activation and telomere elongation. *Journal of Cellular Physiology* **223**, 757-763.

Ziccardi P, Nappo F, Giugliano G, Esposito K, Marfella R, Cioffi M, D'Andrea F, Molinari AM & Giugliano D. (2002). Reduction of Inflammatory Cytokine Concentrations and Improvement of Endothelial Functions in Obese Women After Weight Loss Over One Year. *Circulation* **105**, 804-809.

Ziegelhoeffer T, Fernandez B, Kostin S, Heil M, Voswinckel R, Helisch A & Schaper W. (2004). Bone Marrow-Derived Cells Do Not Incorporate Into the Adult Growing Vasculature. *Circulation Research* **94**, 230-238.

Zieman SJ, Melenovsky V & Kass DA. (2005). Mechanisms, Pathophysiology, and Therapy of Arterial Stiffness. *Arteriosclerosis, Thrombosis, and Vascular Biology* **25**, 932-943.

Chapter 9 Appendices

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Biological Sciences Faculty Research Ethics Committee
University of Leeds

18th November 2010

Dear Karen

Title of study:	**The impact of high intensity exercise upon EPC number and function in young women**
Ethics reference:	**BIOSCI 10-007**
Amendment number:	**1**
Amendment date:	**10/11/10**
Amendment description	**Further clarification**

The above amendment was reviewed by the Biological Sciences Faculty Research Ethics Committee at its virtual meeting on 16th November 2010.

The following documentation was considered:

Document	*Version*	*Date*
BIOSCI_10-007_Committee_response birch.doc	1	10/11/10
Ethical Review Form harris.docx	2	10/11/10
patient_info_sheet1harris.doc	2	10/11/10
consent form high intensity women.doc	2	10/11/10

On the basis of the information provided, the Committee approves this project.

Yours sincerely

Jennifer Blaikie
Research Ethics Administrator,
Research Support
On Behalf of Professor Eric Blair
Chair, Biological Sciences FREC

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Biological Sciences Faculty Research Ethics Committee
University of Leeds

23 October 2013

Dear Mark

Title of study: **The effect of high intensity exercise on endothelial progenitor cell numbers and function in post-menopausal women.**

Ethics reference: **BIOSCI 10-016**

I am pleased to inform you that the above research application has been reviewed by the Biological Sciences Faculty Research Ethics Committee and following receipt of the amendments requested, I can confirm a favourable ethical opinion on the basis described in the application form and supporting documentation as of the date of this letter.

The following documentation was considered:

Document	*Version*	*Date*
BIOSCI 10-016 researcher's response.docx	1	07/03/11
BIOSCI 10-016 Ethical_Review_Form_V3 high intensity post-menopause.doc	1	14/02/11
BIOSCI 10-016 information form high intensity post menopausal.docx	1	14/02/11
BIOSCI 10-016 consent form high intensity post menopausal.docx	1	14/02/11

Please notify the Committee if you intend to make any amendments to the original research as submitted at date of this approval. This includes recruitment methodology and all changes must be ethically approved prior to implementation.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited.

Yours sincerely

Jennifer Blaikie
Research Ethics Administrator
Research Support
On Behalf of Professor Eric Blair
Chair, BIOSCI Faculty Research Ethics Committee
CC: Student supervisor(s)

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Biological Sciences Faculty Research Ethics Committee
University of Leeds

Amy Weeks
PhD Research Student
Institute of Psychological Sciences
University of Leeds
Leeds LS2 9JT

23 October 2013

Dear Amy

Title of study:	**The effect of (i) physical activity and (ii) continuous versus intermittent exercise training upon cardiovascular and cognitive function in obese women**
Ethics reference:	**BIOSCI 10-021**
Amendment:	**August 2012**

I am pleased to inform you that the application for the ethical review of an amendment to the above project has been reviewed by a representative of the Biological Sciences Faculty Research Ethics Committee and I can confirm a favourable ethical opinion on the basis of the information provided in the following documents:

Document	*Version*	*Date*
BIOSCI 10-021 Amendment Aug 2012 Amendment_form cognitive[1][1].doc	1	07/08/12
BIOSCI 10-021 Amendment Aug 2012 distribution e-mail amy[1].docx	1	07/08/12
BIOSCI 10-021 Amendment Aug 2012 poster august 2012.pub	1	07/08/12

Please notify the Committee if you intend to make any further amendments to the research as submitted at date of this approval. This includes recruitment methodology and all changes must be ethically approved prior to implementation.

Please note: You are expected to keep a record of all your approved documentation, as well as documents such as sample consent forms, and other documents relating to the study. This should be kept in your study file, which should be readily available for audit purposes. You will be given a two week notice period if your project is to be audited.

Yours sincerely

Jennifer Blaikie
Senior Research Ethics Administrator
Research & Innovation Service
On Behalf of the BIOSCI Faculty Research Ethics Committee

CC: Student supervisor(s)