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The Impact of Blood Flow Restricted Exercise on the Peripheral Vasculature

by
Julie Hunt

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of
Doctor Philosophy of Loughborough University

October 2013

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Abstract

Distortion to hemodynamic, ischemic and metabolic stimuli during low load resistance exercise with blood flow restriction (BFR) may influence regional vascular adaptation. This thesis investigated the acute response and chronic adaptations of the peripheral vasculature to low load resistance exercise with BFR. The methodology utilised Doppler ultrasound, strain gauge plethysmography and muscle biopsy for insightful measures of the vasculature at different regions of the arterial tree. Short term (4-6 weeks) localised low load (30-40% 1RM) resistance exercise with BFR increased brachial (3.1%) and popliteal (3.3%) artery maximal diameter (in response to ischemic exercise), forearm (29%) and calf (24%) post-occlusive blood flow, and calf filtration capacity (14%). These findings indicate potential vascular remodelling at the conduit (chapters 3, 4) resistance (chapter 4) and capillary (chapter 4) level of the vascular tree. Regional, rather than systemic, factors are responsible for these adaptations as evidenced by an absent response in the contralateral control limb. Transient improvements in popliteal artery FMD% occurred at week 2 before increased maximal diameter at week 6, suggesting functional changes precede structural remodelling (chapter 4). Maximal brachial artery diameter and forearm post-occlusive blood flow returned to baseline values after a 2 week detraining period, signifying rapid structural normalisation after stimulus removal (chapter 3). Enhanced capillarity, despite low training loads, could be explained by augmentation of VEGF (~7 fold), PGC-1 α (~6 fold) and eNOS (~5 fold) mRNA, and upregulation VEGFR-2 (~5 fold) and HIF-1 α (~2.5 fold) mRNA with BFR (chapter 5). This indicates a targeted angiogenic response potentially mediated through enhanced metabolic, ischemic and shear stress stimuli. Large between subject variability in the level of BFR was observed during upper and lower limb cuff inflation protocols. Adipose tissue thickness and mean arterial pressure were the largest independent determinants of upper and lower limb BFR, respectively (Chapter 6). In conclusion, this thesis demonstrates that low load resistance exercise with BFR induces adaptation in the conduit, resistance and capillary vessels. The mediators of this response are likely to be the hemodynamic and chemical signals elicited by repeated bouts of BFR resistance exercise, although confirmation of these mechanisms is required. The functional significance of these adaptations is unknown and warrants further investigation.

Key words

Blood flow restriction

Resistance exercise

Ischemia

Flow mediated dilation

Vascular function

Arterial remodelling

Angiogenesis

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List of abbreviations

AMPK	adenosine monophosphate activated protein kinase
AT	adipose thickness
ATP	adenosine triphosphate
BFR	blood flow restricted/restriction
BMI	body mass index
CO ₂	carbon dioxide
CON	control
CSA	cross sectional area
Ct	cycle threshold
DBP	diastolic blood pressure
DC	dilatory capacity
DNA	deoxyribonucleic acid
d/wk	days per week
EC	endothelial cell
ECG	electrocardiograph
EDHF	endothelium-derived hyperpolarizing factor
ERR- α	estrogen-related receptor- α
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cell
FMD	flow mediated dilation
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
h	hours
H ⁺	hydrogen ions
HIF	hypoxia-inducible factor
K ⁺	potassium ion
K _f	filtration capacity
L-NMMA	L-NG-monomethyl arginine citrate
MAP	mean arterial pressure
MMP	matrix metalloproteinase

mRNA	messenger ribonucleic acid
MVC	maximal voluntary contraction
NO	nitric oxide
NOS	nitric oxide synthase
O ₂	oxygen
PCr	phosphocreatine
PGC-1 α	peroxisome proliferate-activated receptor gamma coactivator 1 α
P _i	inorganic phosphate
PI3 kinase	phosphatidylinositide 3-kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SEM	standard error of the mean
SEE	standard error of the estimate
SBP	systolic blood pressure
SR _{AUC}	shear rate area under curve
TAMV	time average mean velocity
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VO _{2max}	maximum oxygen uptake
VO _{2peak}	peak oxygen uptake
VSM	vascular smooth muscle
1RM	one repetition maximum

Chapter 1: Introduction

Exercise training can have a direct impact on the vasculature. Observations began in the coronary vasculature of animals (e.g. Wyatt & Mitchell, 1978; Hudlická, 1982; Laughlin, 1995) and investigations have since progressed to the peripheral arteries of humans (e.g. Green *et al.*, 1994; Thijssen *et al.*, 2007; Tinken *et al.*, 2008, 2010). These studies have revealed exercise training can induce structural (angiogenesis & arterial remodelling) and functional vasculature adaptations. Such adaptations can directly modify cardiovascular risk and influence health and performance alike by impacting blood flow delivery and oxygen-diffusing capacity.

Adaptations to exercise training are typically observed in vasculature perfusing metabolising skeletal muscle but can extend to regions less active during exercise involving large muscle groups. These local and systemic vascular adaptations have been observed with aerobic endurance-type exercise and to a lesser extent resistance exercise. However, there is a need to investigate the vascular adaptations to new more efficient forms of training. Arguably this could also include training methods which allow the concurrent promotion of strength and endurance adaptations. Low load blood flow restricted (BFR) resistance exercise is a novel training stimulus best known for its ability to illicit similar hypertrophic and strength gains as traditional heavy load resistance exercise (Takarada *et al.*, 2000b; Laurentino *et al.*, 2012) with further improvements in skeletal muscle endurance (Takarada *et al.*, 2002; Loeppky *et al.*, 2005; Sumide *et al.*, 2009; Cook *et al.*, 2010; Kacin & Strazar, 2011). Vascular adaptations, coupled with myofiber growth, may enhance muscle blood flow capacity and gas/metabolite exchange contributing to the increased muscular endurance following BFR training. Yet despite a growing interest in this exercise modality the vascular adaptations remain incompletely understood.

1.1 Peripheral arterial tree

Blood flow to the working muscles is determined by both central (cardiac) and peripheral (vascular) influences (Egginton, 2009). The peripheral arterial tree can be divided into three functional classes; conduit, resistance and exchange vessels. In each class of vessel, the structure of the wall is specially adapted to its role, influencing vasodilatory control and mechanisms of adaptation. The conduit vessels are medium in size (0.1-1.0cm in humans) and include the brachial and popliteal artery. Their primary role is to conduct flow from larger elastic arteries to smaller arteries that feed the resistance vessels. The resistance vessels are smaller in size (10-500 μ m) and include narrow terminal arteries and arterioles, which reside within the local tissue. These vessels offer a large resistance to flow and principally regulate local tissue perfusion. The exchange vessels, made up of microvessels and capillaries (4-7 μ m), are the site of substrate delivery and metabolite removal (Levick, 2011).

The wall of conduit and resistance blood vessels consists of three layers; the tunica intima, media and adventitia. The intima is composed of a single layer of endothelial cells (EC) supported by connective tissue. The ECs secrete vasoactive agents nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor (EDHF) and endothelin. The media layer provides mechanical strength and contractile power and is composed of smooth muscle cells embedded in a matrix of elastin and collagen fibres. Vascular smooth muscle (VSM) controls arterial tone by contracting (vasoconstriction) and relaxing (vasodilation) under the control of autonomic nerves and local vasoactive agents. Finally the adventitia is comprised of connective tissue, collagen and elastic fibres and contains fibroblasts, sympathetic nerve fibre terminals and vasa vasorum. In contrast, capillaries are comprised of a single layer of EC, with no VSM cells or adventitial layers, facilitating the rapid passage of solutes (Levick, 2011).

1.1.1 Basal hemodynamics

Blood flow to skeletal muscle is determined by the perfusion pressure and vascular resistance, in accordance with Darcy's law of flow (flow = pressure gradient / resistance). Arterial blood pressure is generally kept within a narrow range by neural reflexes, so local blood flow is largely regulated by changes in vascular resistance (Levick, 2011). Vascular resistance (the reciprocal of conductance) is determined by the viscosity of blood, vascularisation of the tissue (i.e. the size and number of vessels) and the calibre of resistance vessels (Korthuis, 2011). Since the largest pressure gradient occurs across the terminal arteries and arterioles, these vessels are the main site of resistance to blood flow (Clifford, 2011). Blood viscosity is relatively constant over the short term so alterations in skeletal muscle blood flow are regulated by changes in the diameter of resistance vessels. Resistance to flow is inversely proportional to tube radius raised to the fourth power (r^4 ; Poiseuille's Law) so small changes in vessel calibre produce very large alterations in blood flow (Clifford, 2011). This demonstrates the significance of improved vasodilatory function and structural remodelling, which increase arterial lumen size and therefore blood flow capacity.

1.1.2 Vascular tone

The calibre of blood vessels is controlled by the contractile activity of the tunica media VSM. The contractile state of VSM is known as vascular 'tone' and is regulated by central control mechanisms and local factors that are intrinsic to the tissue (Levick, 2011). Intrinsic regulatory mechanisms include myogenic responses, vasoactive metabolites (metabolic vasodilation) and endothelial secretions (flow-mediated dilation), while extrinsic regulation is largely mediated by sympathetic control (Korthuis, 2011). Each of these mechanisms plays an important role in the control of muscle blood flow at rest and during exercise. However the relative importance of vascular control mechanisms is different at various levels of the arterial tree (Figure 1.1).

The myogenic response occurs in smaller arteries and arterioles and is characterised by the constriction/vasodilation of the vessel to increased/decreased transmural pressure (Schubert & Mulvany, 1999). VSM in resistance arteries maintains a degree of contraction, or myogenic

tone at normal intramuscular pressure which allows changes in vessel calibre to occur in either direction in response to vasodilator and vasoconstrictor stimuli (Korthuis, 2011).

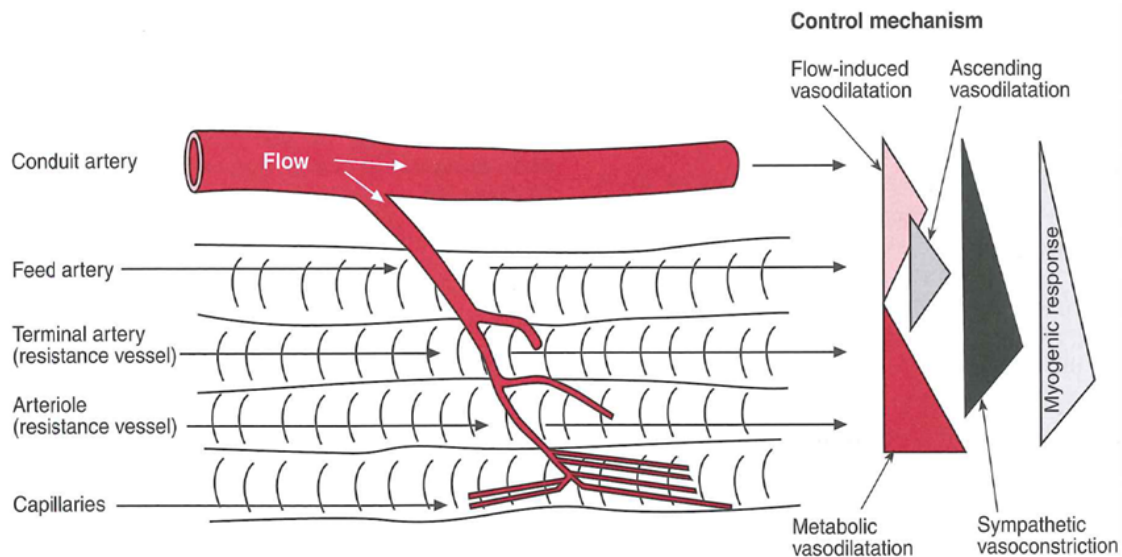


Figure 1.1 Different control of the arterial tree. Taken from Levick (2011).

As muscle metabolism increases with exercise, there is a proportional release of vasodilator metabolites (e.g. adenosine, acidosis, K^+ , CO_2 , ATP) into the interstitial space (Korthuis, 2011). These agents act locally on the resistance vessels causing vasodilation, reduced vascular resistance and increased blood flow. All vasodilatory stimuli can act directly on VSM, converging on the activation of the K_{ATP} channels, the opening of which results in VSM hyperpolarisation, decreased calcium influx and vasodilation (Gielen *et al.*, 2010). The sensitivity to metabolites increases from large down to pre-capillary resistance vessels.

The endothelium can detect chemical substances within the blood as well as physical forces induced by blood flow and changes in transmural pressure (Korthuis, 2011). In response to these chemical and mechanical stimuli the EC release substances (NO, EDHF, prostacyclin, endothelin-1), which act on underlying VSM (Clifford, 2011). The best-characterised vasodilator is NO, generated from L-arginine by endothelial NO synthase (eNOS). Shear stress, the tangential force exerted by flowing blood, provides an important tonic drive for eNOS activation and NO production. Emerging literature also suggests neuronal NOS-derived NO plays an important role in basal vascular tone (Seddon *et al.*, 2008; Melikian *et al.*, 2009).

During exercise, as the resistance vessels undergo metabolic vasodilation, blood flow through the conduit and feed arteries increases. This enhances shear stress, stimulating the production of NO and mediating flow-induced dilation of the conduit artery which serves as a mechanism to augment flow delivery to dilated arterioles. By diffusion, the short lived NO reaches the VSM in the media and causes relaxation via cyclic guanosine monophosphate pathways (Michel & Vanhoutte, 2010).

Feed arteries exhibit the property of ascending or conducted vasodilation. This describes the vasodilation originating in the capillaries that spreads in a proximal direction along the vessel wall by hyperpolarisation, and involves the direct coupling between EC and VSM (Clifford & Hellsten, 2004). Conducted vasodilation occurs in response to muscle contraction and is dependent on NO and ATP-sensitive K^+ channels. The ability of vasodilation to ascend into feed arteries is essential to achieving peak levels of muscle blood flow (Bagher & Segal, 2011).

The highest level of control of vascular tone is extrinsic regulation by sympathetic and parasympathetic vasomotor nerves (Levick, 2011). Sympathetic vasoconstrictor fibres are the most widespread and more dominant on proximal resistance vessels. Sympathetic fibres are tonically active and contribute to basal vascular tone. Reduced sympathetic activity elicits vasodilation while increased sympathetic activity elicits vasoconstriction, raises peripheral resistance and reduces local blood flow.

The variation in vasomotor responsiveness along the arterial tree may account for the heterogeneous adaptation to training (Green *et al.*, 2004b).

1.1.3 Solute exchange

Blood flow capacity of the muscle does not depend on capillary density. Although capillaries offer some resistance to blood flow they contribute less than the terminal arteries and arterioles. The primary effect of a high capillary density is increased endothelial surface area available for gas and nutrient exchange. Fluid movement across the capillary bed in skeletal

muscle, according to the Starling equation, is the product of vascular wall permeability, the surface available for exchange and the gradients of hydrostatic and oncotic pressures across the vascular wall (Charles *et al.*, 2006).

1.2 Vascular adaptations to exercise

Exercise training can induce structural (angiogenesis & arterial remodelling) and functional vasculature adaptations, which can have a positive impact on both health and performance. Improvements in vascular function and outward remodelling of the arterial lumen contribute to the cardio-protective effects of exercise (Thijssen *et al.*, 2012). Endothelial dysfunction is a critical element in the pathogenesis of atherosclerosis (Davignon & Ganz, 2004) and hypertension, and can lead to complications in arteries of the upper and lower limbs. Devising exercise interventions, which sustain or restore a normal/healthy endothelial phenotype in these peripheral arteries, is therefore clinically relevant.

The enlargement of conduit and resistance vessels through improvements in vasodilatory function and/or arterial remodelling can enhance muscle perfusion and vascular conductance during submaximal and maximal exercise (Walther *et al.*, 2008). Improvements in blood flow delivery may enhance aerobic capacity since structural enlargement of the femoral artery is positively associated with changes in leg $\text{VO}_{2\text{peak}}$ (Miyachi *et al.*, 2001), while a strong relationship exists between maximal lower leg vascular conductance and $\text{VO}_{2\text{max}}$ (Snell *et al.*, 1987). However, improvements in $\text{VO}_{2\text{max}}$ are more likely a function of capillarisation (angiogenesis) which provides an increased surface area for gas exchange and reduced diffusion distance (Roca *et al.*, 1992; Prior *et al.*, 2004). Whilst the limiting factor in whole body oxygen uptake is centrally (cardiac output) governed (Saltin & Calbet, 2006), localised peripheral vascular adaptations (vasodilatory capacity & capillarisation) continue to have an influence on endurance performance and functional capacity in elite (Coyle *et al.*, 1988) and clinical populations (Robbins *et al.*, 2011).

The magnitude and location (conduit, resistance, capillary vessel) of vascular adaptation are dependent on the intensity, volume and mode of training (Green *et al.*, 2011). The majority of studies conduct training interventions primarily involving whole body aerobic and sometimes resistance type exercise. The observed adaptations are therefore a consequence of systematic hemodynamic and neuronal stimuli. In contrast, small muscle group training interventions provide insight into the localised effects of exercise without marked changes in central hemodynamic or neural activation (Thijssen *et al.*, 2010). Training models involving single arm and leg exercise have been used previously to assess the impact of localised muscle activity on vascular adaptations (Miyachi *et al.*, 2001; Alomari *et al.*, 2001; Heffernan *et al.*, 2006). A favourable within-measures design can be adopted by performing unilateral exercise training with contralateral comparisons which excludes the substantial variability associated with between-subject differences in genetic vascular capacities.

1.2.1 Conduit artery

Conduit artery function

Conduit artery function is commonly measured by Doppler ultrasound using the flow mediated dilation (FMD) technique. FMD quantifies the vasodilation of a conduit artery following an increase in shear stress, induced by reactive hyperaemia, in response to 5-min distal cuff occlusion. FMD is predominately NO mediated as responses in the radial (Mullen *et al.*, 2001), brachial (Doshi *et al.*, 2001) and femoral (Kooijman *et al.*, 2008) arteries are absent or severely blunted during infusion of the NOS blocker L-NMMA. FMD can therefore provide an index of conduit artery endothelium-dependent NO function (Parker *et al.*, 2008). The reactive hyperaemia shear stimulus is a critical determinant of the FMD response (Pyke & Tschakovsky, 2007) and should be quantified on an individual basis, with area under the shear rate curve (SR_{AUC}) computed from the point of cuff deflation to the time of peak arterial dilation. Normalisation is achieved by dividing the percentage of FMD by SR_{AUC} , but should only be performed if baseline measures correlate (FMD% and SR_{AUC}) and data is normally distributed (Harris *et al.*, 2010).

In cross-sectional observations athletes do not always exhibit enhanced conduit artery function compared to matched non-athletic controls. This is evident in the case of elite rowers, paddlers, runners and cyclists who display similar if not lower femoral artery FMD% compared to healthy match control subjects (Green *et al.*, 2012; Rowley *et al.*, 2012). This is labelled as the ‘athlete paradox’ and is thought to be a consequence of the time course of functional versus structural adaptation (Green *et al.*, 2012). This has highlighted the need for intervention studies to conduct longitudinal measures of FMD when considering conduit artery adaptations to exercise training.

Intervention studies have demonstrated changes in conduit artery endothelial function in response to whole body exercise (Green *et al.*, 2004b; Tinken *et al.*, 2008) and localized moderate intensity dynamic (Hornig *et al.*, 1996; Allen *et al.*, 2003; Tinken *et al.*, 2010) and isometric (McGowan *et al.*, 2007) handgrip exercise. The increase in FMD can occur after just 1-2 weeks but is transient and declines to pre-training values soon after (Tinken *et al.*, 2008, 2010). This is often followed by structural remodelling of the conduit artery (Tinken *et al.*, 2008, 2010) but not always (Birk *et al.*, 2012).

Changes in conduit artery function may be mediated by hemodynamic forces acting on the vessel wall. Enhanced blood flow during exercise increases shear stress, a frictional force that acts tangential to the endothelial cell surface. Shear stress is sensed by the endothelial glycocalyx which activates PI3 kinase and induces Akt-mediated phosphorylation of eNOS at serine 1177 & 1179 (Sessa, 2004; Michel & Vanhoutte, 2010). This enhances eNOS activation and consequently NO production (Sessa, 2004). Repeated exposure to this shear stress stimulus is thought to alter endothelial cell phenotype, increasing eNOS protein expression and NO bioavailability (Laughlin *et al.*, 2008). Hambrecht *et al.* (2003) was the first to demonstrate that changes in endothelial vasodilator function (of the internal mammary artery) following exercise training in humans were closely associated with shear stress-induced eNOS protein expression. Tinken *et al.* (2010) later demonstrated the pivotal role of shear in determining conduit artery adaptation to localised exercise training. Applying a distal forearm cuff during handgrip training attenuated exercise induced shear rate (by >50%) and prevented functional change (FMD) of the brachial artery (Tinken *et al.*, 2010). The magnitude of the

shear rate stimulus induced by exercise therefore has clear implications on conduit artery adaptations to training.

Shear stress is not only influenced by blood flow but is also a function of artery radius as described by the following equation; $SS = 4\eta\dot{Q}/\pi r^3$ (where SS is shear stress, \dot{Q} is blood flow, η is viscosity, and r is the radius of the artery) (Laughlin *et al.*, 2008). Subsequently structural enlargement of a vessel is thought to normalize shear stress level, which removes the stimulus for continued improvement in NO-mediated endothelial function. This would account for the decline in FMD observed after just 2 weeks of training (Tinken *et al.*, 2008, 2010) and the absent enhanced endothelial function in long-term trained athletes (Green *et al.*, 2012). These findings underline the need to monitor changes in shear stress and conduit artery diameter in response to exercise training in order to explain any changes in FMD.

The pattern of flow and shear may also impact endothelial adaptation. The modality and intensity of exercise can influence the frequency and magnitude of pulsations (unidirectional) and oscillations in flow (antegrade [forward] and retrograde [backward] flow), modulating the shear stress pattern on the ECs (Green *et al.*, 2011). In vitro oscillatory shear conditions are found to increase NO production and eNOS expression but also reactive oxygen species (ROS) production (Harrison *et al.*, 2006; Laughlin *et al.*, 2008). Scavenging by free radicals increases NO degradation and reduces bioavailability (Schiffrin, 2008). Correspondingly, FMD is acutely impaired in a dose-response manner to exposures (30min) in retrograde flow (Thijssen *et al.*, 2009). However, oscillatory shear may not be as detrimental when evoked during short bouts of exercise (Laughlin *et al.*, 2008). Indeed, cycling induces oscillatory shear conditions in the brachial artery and is associated with increased eNOS generated NO release (Green *et al.*, 2005) and enhanced FMD in adaptation to training (Tinken *et al.*, 2008). On this basis it would be of interest to monitor conduit artery function in adaptation to localised exercise with modified patterns of flow.

Finally, ECs are activated through exposure to circumferential stress (strain) caused by distension of the arteries due to increased blood pressure (Laughlin *et al.*, 2008). Data on this stimulus is largely obtained from in vitro investigations due to the difficulty in isolating the impacts of changes in pressure from those in flow and shear stress in vivo. Whilst circumferential strain can upregulate eNOS (Cheng *et al.*, 1998; Ziegler *et al.*, 1998), changes

are associated with increased expression of adhesion molecules and ROS, which may reduce NO bioavailability (Harrison *et al.*, 2006). However, much of this data is based on cell exposure to prolonged circumferential strain (24 h/day), rather than the brief increases in blood pressure during exercise which could induce a beneficial change in EC phenotype (Laughlin *et al.*, 2008).

Overall, these studies suggest that shear stress is a key stimulus for local exercise-induced changes in conduit artery function. These adaptations appear to be influenced by both the magnitude and pattern of shear rate. Finally, changes in conduit artery function may be superseded by structural remodelling but to confirm this intervention studies must monitor both indices longitudinally.

Conduit artery structure

Conduit artery remodelling in response to training is often inferred from Doppler ultrasound measures of resting diameter (Rakobowchuk *et al.*, 2005; Zoeller *et al.*, 2009). However, this is considered a poor index of conduit structure given its dependency on basal vascular tone, which is regulated by numerous competitive vasodilators and constrictors, both extrinsically (sympathetic control) and intrinsically (myogenic, endothelial factors, and metabolic by-products) (Naylor *et al.*, 2005). By exposing the conduit artery to a maximal dilatory stimulus the functional influences become obsolete. Traditionally, this has been evoked through sublingual administration of pharmacological vasodilators (glyceryl trinitrate, nitroglycerin) (Schroeder *et al.*, 2000; Green *et al.*, 2004b). However, 5-min ischemic exercise may be used to elicit a similar conduit artery dilatory response (Naylor *et al.*, 2005) and one that is largely NO independent (Mullen *et al.*, 2001). This provides a more valid, albeit surrogate, measure of conduit vessel structure. As a result, this ischemic exercise stimulus is now frequently employed (Tinken *et al.*, 2008, 2010; Birk *et al.*, 2012) and often referred to as a dilatory capacity (DC) test.

Arterial remodelling refers to the enlargement of pre-existing arterial vessels (Prior *et al.*, 2004; Semenza, 2007a) and is observed in the conduit artery with exercise training. Cross-sectional observations confirm that athletes have larger vessel diameters than matched non-

athletic controls (Huonker *et al.*, 2003). These adaptations are localised to the predominantly trained limb as larger femoral and brachial arteries are found in elite athletes engaged in lower (runners and cyclists) and upper (canoe paddlers and spinal cord injured athletes) body exercise, respectively (Rowley *et al.*, 2012). While elite squash players have a larger brachial artery in their dominant (racquet-arm) compared to non-dominant arm (Rowley *et al.*, 2011). These findings suggest remodelling of the arterial lumen is largely driven by local mechanisms. This is reinforced by longitudinal training studies that note enlarged conduit artery diameter in the trained limbs only (Miyachi *et al.*, 2001; Dinunno *et al.*, 2001).

The time course of conduit arterial remodelling has been established by two investigations in response to whole body (cycling) and local exercise (handgrip) training (Tinken *et al.*, 2008, 2010). Brachial artery vasodilator response to ischaemic exercise displayed a gradual increase throughout both 8-week exercise-training interventions. More importantly, Tinken *et al.* (2008) observed that these changes to conduit artery structure began after functional (FMD%) changes had peaked and continued as FMD returned to baseline over the progressive weeks. This is consistent with the hypothesis that the artery enlarges to mitigate the increase in shear stress brought about by repeated exercise bouts (Thijssen *et al.*, 2010), and that this remodelling process continues until shear stress has been restored to baseline (Tuttle *et al.*, 2001) and in doing so removes the stimulus for further functional adaptation. By attenuating the shear stress stimulus during handgrip training (distal cuff inflation) changes in brachial artery dilatory capacity are abolished, confirming the significance of shear rate in localised conduit artery remodelling (Tinken *et al.*, 2010).

Expansive remodelling in response to repeated increases in blood flow/shear stress involves a sequence of events in the arterial wall that are NO-dependent (Rudic *et al.*, 1998). The activation of the EC by elevated shear stress causes proliferation and an increased expression of adhesion molecules and chemotactic factors (Silver & Vita, 2006). This leads to the accumulation of leucocytes (monocytes and T-lymphocyte), which increase the expression of matrix metalloproteinase (MMPs) stimulating the break down of the extracellular matrix (Pasterkamp *et al.*, 2004). Local expression of growth factors contribute to proliferation and migration of VSM cells (Pipp *et al.*, 2003; Heil & Schaper, 2004; Hoefer *et al.*, 2013). NO-dependent signalling is fundamental for processes of arterial remodelling (Rudic *et al.*, 1998),

including the activation of MMPs (Tronc *et al.*, 2000), the production of vascular endothelial growth factor (VEGF) (Yu *et al.*, 2005) and the regulation of endothelial and smooth muscle cell proliferation and migration (Sessa, 2004). Blocking NOS (via L-NMMA) prevents arterial enlargement in spite of high flow conditions (Tronc *et al.*, 1996) demonstrating the central role of NO in the remodelling process. Overall, these studies suggest that arterial remodelling involves a shear stress response with transient activation of pro-inflammatory signalling pathways.

1.2.2 Resistance vessel

Peripheral resistance vessel function and structure is typically assessed using strain gauge plethymography measures of limb blood flow (Wilkinson & Webb, 2001). Assessment of resistance vessel function involves the intra-arterial infusion of endothelium-dependent (acetylcholine) and independent (sodium nitroprusside) agonists for stimulated NO release, or NOS antagonists (L-NMMA) for examination of basal endothelial function (Wilkinson & Webb, 2001). The resultant vasodilatory (NO agonists) or vasoconstrictor (NOS antagonists) response is detected by plethysmographic measurement of change in limb volume. However, the need for arterial cannulation makes this method unsuitable for most non-clinical settings.

Alternatively, studies have quantified reactive hyperaemia in response to a period of ischemia (5-min) as an index of resistance vessel function (Berrazueta *et al.*, 2010). The limitation of this method is the inability to elucidate mediators of the vasodilatory response which is largely NO-independent (Engelke *et al.*, 1996) but influenced by myogenic and metabolic (prostaglandins, adenosine) phenomenon (Carlsson *et al.*, 1987) and resistance vessel structure (Joyner *et al.*, 2001). The latter is more accurately determined from the peak blood flow response to maximal vasodilatory stimuli such as prolonged arterial occlusion (10-min) (Sinoway *et al.*, 1987; Green *et al.*, 1994) or ischemic exercise (Naylor *et al.*, 2005). These responses are not influenced by sympathetic tone (Takeshita & Mark, 1980) or administration of vasodilators (Naylor *et al.*, 2005) and therefore represent the minimum forearm vascular resistance, which is a function of total resistance vessel cross sectional area (CSA) (Wilkinson & Webb, 2001). More recently, conduit artery (maximal diameter) and resistance vessel (peak

blood flow) structure have been assessed simultaneously using Doppler ultrasound in response to ischemic exercise (Naylor *et al.*, 2005).

It has long been recognised that physical training increases the capacity for vasodilation in the active limbs. Snell *et al* (1987) noted the reactive hyperaemia produced by ischemic exercise to fatigue (~ 2min) was 19-fold higher in trained versus sedentary men. Further observations by Sinoway *et al* (1986) suggested a localised peripheral response to training with the preferred arms of tennis players exhibiting a much higher (42%) peak vasodilator response to 5-min ischemic exercise compared to the non-preferred limb. A maximal vasodilatory stimulus was used in both the aforementioned studies, results therefore reflect maximal vascular conductance, indicative of resistance vessel structural adaptation.

Changes in reactive hyperaemic blood flow are observed in response to localised small muscle group exercise training (Sinoway *et al.*, 1987; Green *et al.*, 1994; Alomari *et al.*, 2001). Four weeks of high intensity handgrip training (~70% maximal voluntary contraction [MVC], 20-30 min, 4-5 d/wk) increased peak blood flow response to 5-min ([20.6%] Alomari *et al.*, 2001) and 10-min periods of ischemia ([23%] Green *et al.*, 1994; [30%] Sinoway *et al.*, 1987). Moreover, Green *et al* (1994) did not observe any change in either endothelium-dependent or independent NO vasodilatory function (4 weeks) suggesting the enhanced resistance vessel vasodilatory capacity was structurally mediated. This adaptation may occur over a rapid time course with improvements in reactive hyperemic blood flow (5-min ischemia) evident after just 1 week of handgrip training (60% MVC) and plateauing thereafter (Alomari & Welsch, 2007; Alomari *et al.*, 2010). However, investigators were unable to identify the precise mechanism behind this change in reactive hyperaemia as endothelial function (Meredith *et al.*, 1996), myogenic, metabolic (Carlsson *et al.*, 1987) and structural factors (Joyner *et al.*, 2001) may contribute to its response. A more recent study by Tinken *et al* (2010) utilised a greater vasodilatory stimulus (ischemic exercise) and demonstrated a gradual increase in peak reactive hyperaemia from week 2 of 8 weeks moderate intensity handgrip training. Collectively, results suggest short term local exercise training of moderate to high intensity can induce structural remodelling of the resistance vessels and that this adaptation occurs earlier than that seen in the conduit artery.

Despite the recognition of resistance vessel remodelling in response to exercise training, the signals that initiate this adaptation are poorly understood. The improvements in reactive hyperaemia occur in the trained limb only (Sinoway *et al.*, 1987; Green *et al.*, 1994; Alomari *et al.*, 2001), implying that local changes in haemodynamics may be responsible. The rate of luminal expansion, remodelling events within the wall layers (cell proliferation & migration), and the degree of eNOS gene expression are influenced by the level of blood flow alteration induced by ligation of rat mesenteric arteries (Tuttle *et al.*, 2001). This suggests resistance vessel remodelling is regulated by shear stress NO-dependent mechanisms. In support, the application of a distal forearm cuff during handgrip training, which attenuated exercise induced shear rate (by >50%), prevented improvements in peak reactive hyperaemia that were observed in the non-occluded contralateral exercise control (Tinken *et al.*, 2010). The growth of resistance vessels further downstream (pre-capillary) however, may be stimulated by pressure or circumferential wall stress (stretch) rather than increased shear stress (Price & Skalak, 1994). Mechanical compression of the vessels due to skeletal muscle contraction may also signal remodelling in skeletal muscle (Chen *et al.*, 2002). The stretch and compression of the ECs stimulate eNOS activation and therefore likely mediates arterial remodelling through NO-signalling pathways.

In addition to mechanical forces, the closer proximity of the resistance vessels to the active skeletal muscle means chemical factors may play a more prominent role in vascular control, and potentially the adaptive process. Metabolic vasodilators (NO, adenosine, ATP, K⁺, lactate, CO₂) released by the contracting muscle, bind to receptors located on the VSM stimulating relaxation and stretch of the ECs (Korthuis, 2011). Metabolic stress triggering the breakdown of ATP will stimulate AMP kinase (AMPK) to phosphorylate eNOS on Ser 1177 (Sessa, 2004; Michel & Vanhoutte, 2010). This is the same signalling transduction pathway (post-translational modification) activated by shear stress (Hambrecht *et al.*, 2003). Arterial remodelling of the resistance vessels may be further facilitated by purinergic signalling since ATP and its breakdown product, adenosine, regulate smooth muscle and endothelial cell proliferation (Burnstock, 2009).

1.2.3 Capillaries

Skeletal muscle capillarity can be non-invasively assessed using venous congestion plethysmography. The technique involves the application of small cumulative (venous occluding) pressure steps, which increase limb volume (forearm or calf) as a result of venous filling and fluid filtration (Gamble *et al.*, 1993). The latter enables the calculation of capillary filtration capacity (K_f), which is determined by Starling's forces and therefore dependent on microvascular wall surface area and permeability (Brown *et al.*, 2001). Microvascular surface area is expected to increase as a result of capillary growth, enhancing fluid filtration. Charles *et al.* (2006) confirmed changes in K_f were strongly associated with direct measures of the length of capillary-to-fibre contact following endurance training, allowing the latter to be used as an index of capillarity. Direct measures of capillarisation require a large muscle biopsy and analysis by immuno-histochemistry to determine capillary density and/or capillary-to-fibre ratio. Alternatively the angiogenic potential of an exercise bout can be determined by gene and protein analysis requiring only a small micro biopsy. The acute gene expression of angiogenic factors can inform chronic adaptations of capillary growth with training (Hellsten *et al.*, 2008; Høier *et al.*, 2010; Hoier *et al.*, 2012).

The extent of skeletal muscle capillary growth is dependent on the type, intensity and duration of exercise training. Cross-sectional observations report increased calf capillary density (Saltin *et al.*, 1995) and K_f in endurance trained athletes, whereas strength-trained athletes have values similar to sedentary controls (Brown *et al.*, 2001). Increases in muscle capillarity with resistance exercise training are often proportional to changes in fibre size (McCall *et al.*, 1996; Green *et al.*, 1999) and seldom match those seen in response to endurance training. The onset of capillary growth further depends on the intensity of exercise, with capillarisation evident after high intensity endurance (70-80% $\text{VO}_{2\text{max}}$, Denis *et al.*, 1986) and interval training (90% & 150% leg $\text{VO}_{2\text{max}}$, Jensen *et al.*, 2004) but absent after lower intensity regimes (45% $\text{VO}_{2\text{max}}$, Schantz *et al.*, 1983). The time-course of capillary growth was originally identified in rat gastrocnemius muscle, with an increase in venular and arteriolar capillary density after 1-2 week of exercise training (Suzuki *et al.*, 2001). The response may be slower in human skeletal muscle as capillarisation is absent at 2 weeks but evident after 4 weeks of high intensity aerobic training (Jensen *et al.*, 2004).

Angiogenesis is the term given to describe the growth of new capillaries from pre-existing blood vessels (Egginton, 2011). It is a process largely coordinated by chemical factors released from endothelial, smooth muscle and skeletal muscle cells (Adair & Montani, 2010). EC activation is the first step in the angiogenic process and occurs with the binding of VEGF to its principle EC-specific tyrosine kinase receptor VEGFR-2 (Milkiewicz *et al.*, 2005). VEGF is the central growth factor in angiogenesis as activation (binding to VEGFR-2) induces capillary permeability and stimulates EC proliferation and migration (Neufeld *et al.*, 1999). VEGF activation of the VEGFR-2 is crucial in this process because exercise-induced angiogenesis is abolished in skeletal muscle in which the receptor is inhibited (Lloyd *et al.*, 2005; Milkiewicz *et al.*, 2005). However, a coordination of events must follow this activation for the angiogenic process to continue. An important step for the progression of angiogenesis involves the degradation of the basement membrane and extracellular matrix (Prior *et al.*, 2003). This is primarily mediated by MMPs and activated by mechanical stretch of the skeletal muscle. This facilitates the migration of ECs allowing capillary sprouting (Brown & Hudlicka, 2003). However, angiogenesis may also proceed by disruption of the luminal glycocalyx of EC as a result of high shear stress (Brown *et al.*, 1996). This stimulates capillary development by longitudinal splitting (where the lumen divides to form two capillaries) and requires the activation of eNOS (Egginton, 2011). NO release also increases capillary proliferation in the early stages of EC activation through the upregulation of VEGFR-2 and VEGF (Milkiewicz *et al.*, 2005).

Increased metabolic activity and/or decreased oxygen availability are thought to be the primary signals that modulate the up-regulation of angiogenic growth factors (VEGF) and their receptors (VEGFR-2) (Adair & Montani, 2010). Activation of the skeletal muscle increases O₂ utilisation and decreases the partial pressure of O₂ in the muscle. The imbalance between O₂ supply and demand activates hypoxic and metabolic signalling pathways. Local skeletal muscle hypoxia stabilises transcription factor hypoxia-inducible factor 1 (HIF-1 α) stimulating production of VEGF (Shweiki *et al.*, 1992; Gustafsson *et al.*, 1999; Ameln *et al.*, 2005). Metabolic stress triggering the breakdown of ATP will stimulate AMPK, which can increase VEGF transcription through the activation of peroxisome proliferate-activated receptor gamma coactivator 1 α (PGC-1 α). Mice lacking PGC-1 α in their skeletal muscle failed to increase VEGF expression (Leick *et al.*, 2009) and capillary density (Chinsomboon *et*

al., 2009) in response to exercise training, demonstrating the central role of this transcription co-activator.

In summary, arterial remodelling and angiogenesis are processes of vascular growth that occur in different portions of the vascular tree, which follow a distinct time course and are primarily regulated by different stimuli. Hemodynamic signals initiate improvements in vascular function followed by structural enlargement (arterial remodelling) in the conduit and resistance vessels. While chemical signals activated by local tissue hypoxia and metabolic activity, stimulate the formation of new capillaries from pre-existing vessels (angiogenesis). The vascular adaptations to exercise modes that distort these hemodynamic and chemical signals therefore warrant investigation. Few studies collectively monitor changes at the conduit, resistance and capillary level in response to exercise training or monitor the time course of adaptation at different levels of the arterial tree.

1.3 Resistance exercise with blood flow restriction (BFR)

Tourniquet induced circulatory occlusion has been used for centuries in medical practice to reduce blood loss in emergency situations and create a bloodless surgical field. It has also been used to investigate a wide range of physiological processes such as muscle metabolic regulation (e.g. Greenhaff *et al.*, 1993) and cardiovascular reflex control (e.g. Bull *et al.*, 1989). The application of a pneumatic cuff alongside exercise training was popularized in Japan by Yoshiaki Sato in the mid-1980s. Since then blood flow restricted (BFR) training has evolved to incorporate many forms of exercise, including walking (Abe *et al.*, 2006; Ozaki *et al.*, 2011), cycling (Sundberg, 1994), circuits (Ishii *et al.*, 2005) and resistance training. The latter is used most prevalently in research although there is no consensus on the method of application. Typically, resistance exercise is performed at low loads (20-40% one repetition maximum [1RM]) since no additional benefits are observed at higher loads (Takarada *et al.*, 2000b; Yamada *et al.*, 2004; Wernbom *et al.*, 2006; Laurentino *et al.*, 2008).

A restrictive cuff or band (typically a pneumatic cuff) is placed on the proximal portion of the exercising limb and inflated to a pressure which reduces arterial blood inflow to the working

muscle. Under these conditions venous outflow is suppressed and blood pools as evidenced by increased venous capacity (swelling) distal to the cuff (Iida *et al.*, 2005). A complex blood velocity profile is likely to exist in the artery near the cuff (Figure 1.2) with increased downstream resistance inducing turbulent arterial inflow. This flow velocity (determined via pulse wave amplitude) diminishes proportionately with increasing cuff pressure (Sumide *et al.* 2009).

The required level of arterial blood flow restriction is undefined, but if appropriate should elicit increased muscle activity at the same external load without inducing premature fatigue (contractile/metabolic impairment) (Loenneke *et al.*, 2010). Subsequently, the cuff pressure (mmHg) varies between investigations, with further discrepancy due to differences in compression cuff design (size, material) and exercising limb circumference (upper arm or thigh) (Fahs *et al.*, 2012b). Further investigation is required to measure the degree of restriction imposed with BFR to provide further interpretation of the physiological effects.

1.3.1 Vascular adaptations to BFR exercise

The alteration in normal blood flow, on which this modality is based, may expose the vasculature to distorted hemodynamic and chemical/metabolic signals. This provides a physiological rationale for investigating vascular adaptations. Yet despite this, few studies have considered the impact of BFR training on vasculature. Those that have attempted to do so report mixed findings.

1.3.1.1 Conduit artery

Endothelial function is associated with pulse pressure and pulse wave velocity (McEniery *et al.*, 2006), suggesting any endothelial mal-adaptation (reduced NO bioavailability) would be reflected by measures of increased arterial stiffness and reduced compliance. Interestingly,

short-duration (4-6 weeks) low load BFR exercise training did not negatively influence peripheral pulse wave velocity (Clark *et al.*, 2011) or arterial compliance (Kim *et al.*, 2009; Fahs *et al.*, 2012a). Taken together, this suggests BFR exercise does not negatively impact endothelial function. However, more localised measures of the conduit artery imply otherwise. Credeur et al (2010) found 4 weeks of handgrip training combined with venous restriction (cuff applied proximally on the upper arm) caused superior strength gains but reduced brachial artery FMD compared with the non-restricted trained arm. This occurred despite a significant increase in the SR_{AUC} with BFR training, demonstrating a blunted vasodilatory response to a greater shear stimulus. The protocol however did not replicate typical low load resistance exercise with BFR since it was performed at heavy training loads (60% MVC) over a prolonged period (20min). Restricting blood flow for this duration may induce ischemic-reperfusion injury and contribute to endothelial damage (Kilian *et al.*, 2005; Rakobowchuk *et al.*, 2013). The effect of traditional low load BFR resistance exercise (20-40% 1RM, <10 min) on conduit artery function is unknown and warrants investigation. Changes in conduit artery geometry were also unaccounted for in the investigation by Credeur et al (2010). Larger arteries display a smaller dilatory response to functional stimulation (Schroeder *et al.*, 2000; Thijssen *et al.*, 2008) and therefore structural enlargement could have accounted for the reduced FMD. This needs to be investigated further in response to BFR exercise by conducting measures of conduit artery maximal dilation, as an index of structural capacity, alongside measures of endothelial function (FMD).

1.3.1.2 Resistance vessels

Early reports indicate positive adaptation at the resistance vessel level in response to ischemic and BFR exercise stimuli. Kimura et al (2007) reported increased forearm blood flow response to acetylcholine following 4 weeks of repeated bouts of 5-min ischemia. This suggests improved NO-mediated resistance vessel function in response to a repeated ischemic pre-conditioning, which shares many similarities with the BFR training stimulus. Improvement in endothelial function may precede structural remodelling of the resistance vessels. Patterson & Ferguson (2010) noted an increase in calf reactive hyperaemic blood flow following just 4

weeks of BFR plantar flexion exercise, which was absent in the non-occluded contralateral control. Whilst this could indicate structural enlargement of the resistance vessels, it may also be influenced by metabolic and myogenic factors (Carlsson *et al.*, 1987). However, Fahs *et al* (2012) noted an increase in calf resistance vessel flow without changes in mean arterial pressure or arterial compliance, suggesting probable structural remodelling. Measuring the peak reactive hyperaemic response to a maximal dilatory stimulus such as ischemic exercise should diminish the impact of changes in vessel function and enable quantification of resistance vessel structural adaptation.

1.3.1.3 Capillaries

The potential for BFR exercise training to stimulate capillary growth has long been recognised (Esbjörnsson *et al.*, 1993) and is consistent with hypoxia being the principle stimulus of angiogenesis. Historically, studies have used a pressure chamber to reduce blood flow (by ~15-20% BFR) during single leg exercise (Gustafsson *et al.*, 1999, 2002, 2007; Norrbom *et al.*, 2004). This moderate level of BFR has enabled the performance of prolonged exercise (45 min), which is in contrast to the tolerable durations of 'traditional' BFR resistance exercise (>10min). Evans *et al* (2010) was the first study to investigate a more conventional mode of BFR resistance exercise, reporting a 26% increase in K_f (an indirect measure of capillarization) following 4 weeks of short duration plantar flexion training. However, Evans *et al* (2010) utilised a discontinuous occlusion protocol (deflating the cuff between sets) which may facilitate angiogenesis by enhancing capillary shear stress (Hudlicka & Brown, 2009) through repeated cuff deflation. The capillarisation response to traditional low load BFR resistance exercise (20-40% 1RM, <10 min) with continuous occlusion is unknown. Determining the angiogenic transcriptional response to an acute bout of low load BFR resistance exercise could also inform the potential for capillary growth with BFR training (Gustafsson *et al.*, 2007).

This review is short as a consequence of the few studies considering the impact of BFR training on the vasculature. The differences in BFR exercise protocols, combined with

observations of adaptations at different parts of the arterial tree, prevent a clear understanding of the overall vascular adaptations to this mode of training. Further investigation is clearly warranted as this training modality is gaining in popularity and considered as a viable application in more vulnerable populations e.g. older people. Assessing adaptations throughout the vascular tree in response to the same localised low load BFR training intervention will provide better insight as to the overall adaptations and potential mediators of this response. This should include a more detailed investigation of changes at the conduit artery level, involving both functional and structural measures. Arterial remodelling of the resistance vessels can be clarified by assessing the peak blood flow response to a maximal dilatory stimulus. Finally, the capacity for traditional BFR resistance exercise protocols (with continuous occlusion) to induce angiogenesis can be assessed and the mediators investigated.

1.3.2 Physiological signals during BFR exercise

The physiological rationale for investigating vascular adaptations to BFR resistance exercise relate to the distorted hemodynamic and chemical signals it induces. Having already discussed the importance of these physiological signals in regulating vascular adaptation the hemodynamic and chemical signals during BFR resistance exercise must be considered.

1.3.2.1 Haemodynamic signals

During BFR exercise. During cuff inflation the conduit artery is exposed to mechanical compression and a distorted hemodynamic profile (Figure 1.2). A pressure-dependent increase in retrograde shear is reported in the brachial artery proximal to the cuff likely due to the increased downstream vascular resistance (Thijssen *et al.*, 2009). Performing exercise may alleviate this retrograde component although antegrade flow is likely to remain suppressed, abolishing the increase in brachial artery FMD % post exercise (Tinken *et al.*, 2009, 2010). Intramuscular pressure is increased underlying the cuff causing partial collapse of the artery and a decrease in diameter, which may expose the artery walls to increased shear stress.

Enhanced eNOS expression is reported in ECs exposed to similar high shear regions underlying a cast in animal models (Cheng *et al.*, 2005; Chiu & Chien, 2011).

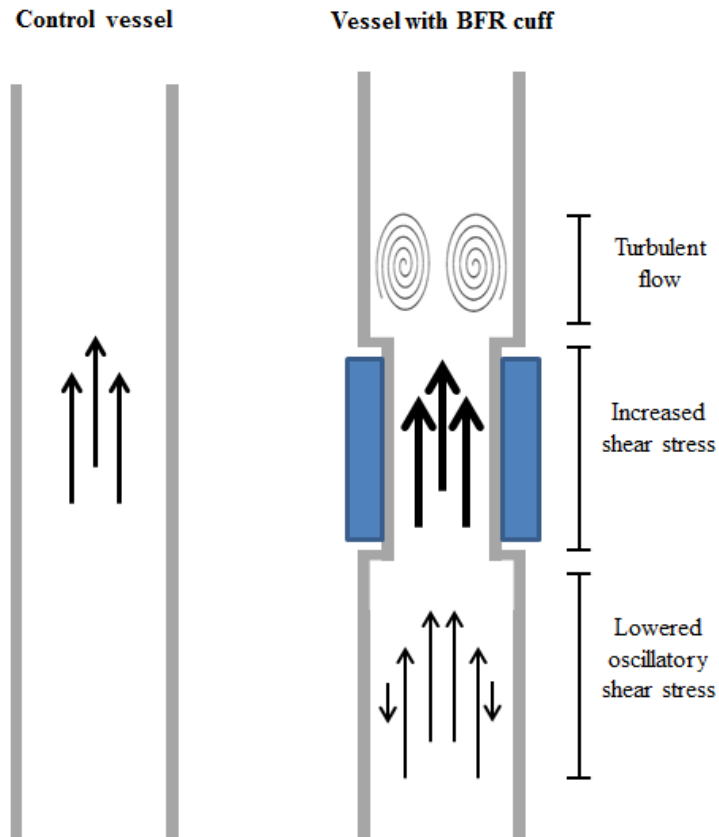


Figure 1.2 A schematic representation of the proposed shear stress pattern in the conduit artery induced by pneumatic cuff inflation. On the left is a control vessel without BFR, which has laminar blood flow (indicated by the parallel arrows). On the right is an artery with BFR. Proximal to the cuff shear stress is reduced and oscillatory in nature. External compression from the cuff reduces the underlying artery diameter, increasing shear stress. A region of disturbed turbulent flow occurs immediately distal to the cuff. Adapted from Chiu & Chien (2011).

Immediately downstream (distal) of the cuff there is likely to be a disturbed region of flow with reciprocating shear stress (Hiatt *et al.*, 1989). Indeed, Credeur et al (2010) noted an increase in retrograde shear at rest which was maintained during exercise alongside enhanced antegrade shear rate. This indicates an oscillatory shear profile downstream of the cuff. Acute

exposure to such conditions may augment endothelial NO release (Green *et al.*, 2005) but prolonged exposure (>20-min) to disturbed flow is associated with increased ROS production, endothelial injury (Jenkins *et al.*, 2013) and dysfunction (Rakobowchuk *et al.*, 2013). Oscillatory shear conditions can also increase adhesion molecules on the ECs (Chappell *et al.*, 1998; Hsiai *et al.*, 2003), activating a local inflammatory response which is involved in the initial stages of both arterial remodelling (expansive remodelling) and atherogenesis (constrictive remodelling) (Silver & Vita, 2006). It is not known what effect the hemodynamic conditions described will have on the conduit artery when applied acutely over a BFR training intervention.

Proximal cuff inflation at moderate pressures can induce venous occlusion causing an elevation in venous pressure. This reduces distal perfusion pressure (pressure gradient), decreasing resistance and capillary vessel flow and shear stress (Hudlicka & Brown, 2009) (Hudlika et al 2009). The increase in capillary hydrostatic pressure enhances transcapillary fluid filtration (Scallan *et al.*, 2010).

Post BFR exercise. Following cuff deflation the conduit artery is exposed to reactive hyperaemia increasing shear stress mediated NO release (Tagawa *et al.*, 1994; Dakak *et al.*, 1998). This blood flow response is evoked following the release of occlusion pressures as low as 50 mmHg and is magnified by a preceding bout of ischemic exercise (Takarada *et al.*, 2000b). Blood flow can remain elevated (2-fold above rest) for more than 1-hour following knee extension exercise with BFR (Gundermann *et al.*, 2012) demonstrating the prolonged nature of this shear stimulus.

1.3.2.2 Chemical signals

The BFR stimulus limits arterial inflow and occludes venous return, causing not only tissue hypoxia but also the deprivation of nutrients and the suppressed clearance of metabolic by-products (Kawada, 2005; Tanimoto *et al.*, 2005). Near infrared spectroscopy measures confirm the magnified and prolonged decrease in muscle oxygen levels during low load

resistance exercise with BFR (Kawada, 2005; Tanimoto *et al.*, 2005). This is associated with enhanced metabolic perturbations in the working muscle (increased phosphocreatine depletion, inorganic phosphate production and decreasing muscle pH) (Suga *et al.*, 2009) and the increased lactate acid response to BFR exercise (Takarada *et al.*, 2000a; Sato *et al.*, 2005; Takano *et al.*, 2005; Reeves *et al.*, 2006). In fact, studies suggest intramuscular metabolic stress during moderate load BFR exercise mimics that of heavy load/high intensity exercise without BFR (Reeves *et al.*, 2006; Krstrup *et al.*, 2009; Suga *et al.*, 2010). The interstitial concentration of metabolites derived from active muscle cells plays an important role in vasodilation of arteriolar VSM (Korthuis, 2011), and is likely enhanced under BFR conditions (Figure 1.3)

Decreased oxygen tension and raised muscle metabolism are the key signals regulating VEGF expression. Exercise induced changes in VEGF (mRNA & protein) are strongly correlated with changes in both HIF-1 α (Gustafsson *et al.*, 1999) and lactate acid (Takano *et al.*, 2005) suggesting the VEGF response to exercise is graded according to hypoxic and metabolic stress. With the latter augmented during BFR exercise an enhanced VEGF response is probable. Indeed, plasma VEGF is increased in response to low load resistance exercise with BFR (Takano *et al.*, 2005), but it is not known if this is due to an increase in VEGF mRNA transcription or from secreted VEGF stores in the skeletal muscle (Høier *et al.*, 2010). Observing the expression of HIF-1 α and PGC-1 α mRNA in response to an acute bout of BFR exercise would indicate the involvement of hypoxic and metabolic signalling pathways. The activation of VEGF signalling pathways plays a key role in angiogenesis (Egginton, 2011) and arterial remodelling (Bloor, 2005; Semenza, 2007a) suggesting potential adaptation to vascular structure in response to BFR training. However, a coordination of events must follow the up regulation of VEGF for remodelling processes to progress. Measuring the expression of eNOS and MMP mRNA in response to an acute bout of BFR exercise would indicate the facilitating actions of shear stress and mechanical stretch of the muscle tissue (Egginton, 2011)

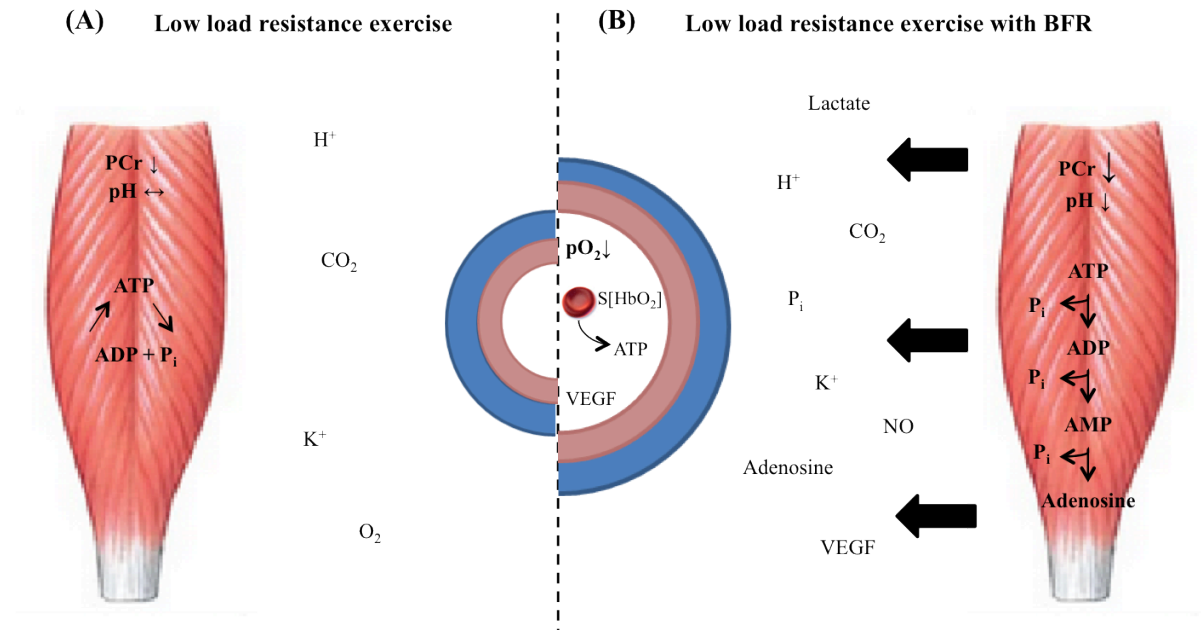


Figure 1.3 The hypothesised metabolic and hypoxic mechanisms of vasodilation during low load resistance exercise with and without blood flow restriction. Adapted from Brown (1995). (A) Exercising at low loads without blood flow restriction induces low levels of metabolic stress [minimal PCr breakdown and no decrease in muscle pH] and arterial vasodilation. (B) Metabolic stress in the skeletal muscle is increased by applying blood flow restriction [increased phosphocreatine (PCr) breakdown, an increase in inorganic phosphate (P_i), a decrease in muscle pH and lactate accumulation]. Enhanced metabolite production and decreased oxygen tension stimulate an arterial vasodilatory response.

1.4 Aims

It is clear that there is limited knowledge on how the peripheral vasculature responds and adapts to BFR resistance exercise and yet there is strong physiological rationale for its investigation. Therefore the main objective of this thesis was to study the impact of BFR resistance exercise on the peripheral vasculature. This was addressed through the following specific research aims:

1. To determine if structural adaptation occurs at the conduit (Chapters 3, 4), resistance (Chapters 3, 4) and capillary (Chapter 4) level of the vascular tree in response to low load resistance exercise with BFR.
2. To determine if changes in conduit artery function precede changes in structural remodelling (Chapter 4).
3. To assess if low load resistance exercise with BFR elicits an angiogenic response and if so identify the potential stimulus/stimuli responsible (Chapter 5).
4. To determine the variation in the level of BFR between participants and establish whether characteristics (i.e. blood pressure, limb size and composition) should be accounted for when prescribing the cuff pressure (Chapter 6).

Chapter 2: General methods

2.1 Participant recruitment

Investigations recruited white Caucasian males and females (18-35 yrs). Each participant completed a general health questionnaire to ensure their suitability to take part (Appendix C). Individuals indicating a history of cardiovascular, metabolic or haematological disorders were excluded from all investigations. Females were excluded from longitudinal investigations (chapter 3-4) because of the natural fluctuation in vascular reactivity during the menstrual cycle (Adkisson *et al.*, 2010). Enhanced blood flow and FMD during the late follicular phase of the menstrual cycle (Adkisson *et al.*, 2010) may have obscured the interpretation of repeated measures, made every 2 weeks, during exercise training. Participants were fully informed of the purpose, risks and discomforts associated with each experiment before providing written, informed consent (Appendices A & B). All studies conformed to current local guidelines and the Declaration of Helsinki and were approved by Loughborough University Ethics Advisory Committee.

2.2 Outcome measures

A number of main outcome measures have been made throughout:

Vascular measures; blood pressure (chapters 3-6), resting conduit artery diameter and blood flow (Doppler ultrasound, chapters 3-6), function (Doppler ultrasound; FMD, chapters 3,4) and structural capacity (Doppler ultrasound; maximal diameter, chapters 3,4), reactive hyperaemia (strain gauge plethysmography & ultrasound; chapters 3,4), capillary filtration (strain gauge plethysmography; chapter 3).

Muscle biopsy measures: angiogenic transcriptional (real-time reverse transcriptase polymerase chain reaction [RT-PCR], mRNA expression) capacity in vastus lateralis muscle samples (chapter 5).

Anthropometric measures; limb circumference (chapters 3-6), subcutaneous adipose tissue thickness (ultrasound, chapter 6)

For all relevant outcome measures, participants were familiarised with testing procedures and training devices during preliminary visits 1 week prior to experimental testing sessions. For Doppler ultrasound measures of conduit artery vessel properties were initially assessed in accordance with the experimental protocol (described below). This established optimal artery image location and familiarised the participant with the occlusion procedures, limiting stress-induced sympathetic activity during the subsequent experimental trial (Harris *et al.*, 2010). To facilitate accurate location of the same arterial segment for repeated measures, digital photographs of the probe position and vessel ultrasound images were taken. During the second preliminary visit, participants were familiarised with the relevant exercise device, 1RM was estimated and a BFR training protocol experienced (chapters 3-6).

2.3 Vascular measures

2.3.1 Doppler ultrasound

Doppler ultrasound imaging was performed by the same sonographer using a Toshiba Powervision 6000 with a multi-frequency linear array transducer (7-11 MHz).

For brachial artery assessments participants lay supine with imaged arm extended and immobilised at an angle approximately 80° from the torso. A 90° B-mode image of the brachial artery at 3 cm depth (11MHz) was obtained >3 cm proximal of the olecranon process.

For popliteal artery assessments participants lay prone with imaged knee flexed at ~20°. A 90° B-mode image of the popliteal artery at 3-4 cm depth (9-11MHz) was obtained behind the popliteal fossa. Images of the popliteal artery were acquired proximal to the branching of the tibial artery at a site either proximal or distal to the branching of the inferior medial genicular artery. This variation between subjects was needed to ensure the highest possible image quality.

B-Mode frequency and gain settings were optimised to achieve an arterial segment with clear intima-media interfaces to facilitate diameter determination (Figure 2.1). In duplex mode, the sample volume was centrally positioned within the artery, the width of the gate minimised and the Doppler beam aligned with direction of flow (insonation angle $\leq 69^\circ$). Spectral Doppler display was optimised by adjustment of receiver gain, dynamic range and pulse repetition frequency (velocity scale). A high-pass Doppler filter was applied to discount arterial wall motion artefacts. Once satisfactory, the ultrasound probe was held in a constant position. A vascular electrocardiograph (ECG) gating module (Medical Imaging Applications, LLC, Coralville, Iowa) triggered acquisition of the ultrasound images on the R-wave pulse of an ECG signal. Sequential end-diastolic images were stored from on-line image digitization.

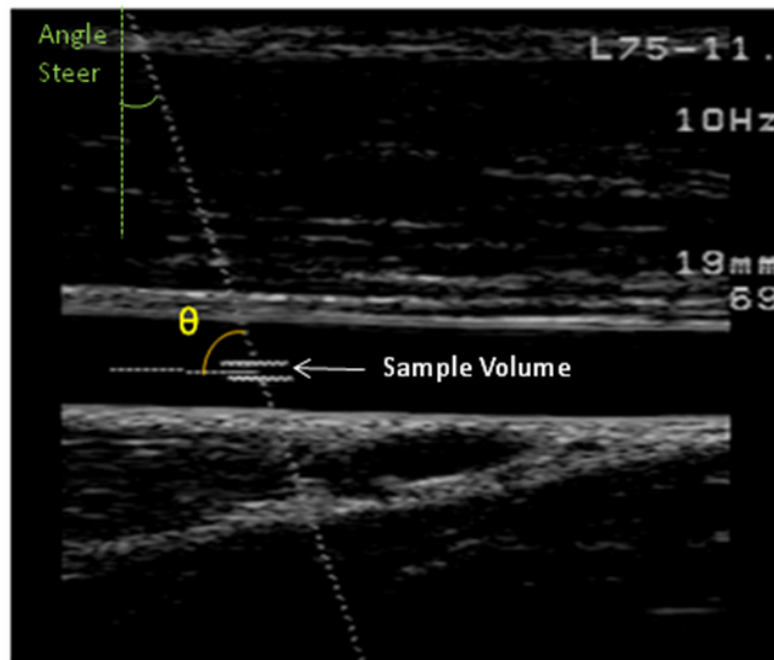


Figure 2.1 Duplex ultrasound; horizontal B-mode image with Doppler beam steered at 20° . Centralised sample volume, 19mm deep. Angle of insonation denotes the cosine between the path of the ultrasound beam and the direction of blood flow, $\theta = 69^\circ$.

Baseline measures

Resting (baseline) conduit artery diameter and flow velocity were recorded for 30 cardiac cycles following 20-min supine/prone rest. Resting arterial diameters were used in the

calculation of normalized and absolute vessel dilation responses to the FMD and DC protocols. Ultrasound settings were standardised for each individual and kept constant for repeated measures. The sonographer had access to the preliminary artery images to ensure identical positioning (of the same arterial segment) for repeated measures using anatomical landmarks which consisted of positioning relative to arterial branching, collateral vessels and connective tissue/muscle striation orientation.

Flow mediated dilation (FMD)

For the ischemic FMD stimulus a pneumatic cuff (E20 Rapid cuff inflator and AG101 Cuff Inflator Air Source, Hokanson, WA, USA) was placed downstream from the ultrasound probe (immediately distal to olecranon process / popliteal fossa) and inflated to supra systolic pressures >200 mmHg. Occlusion was maintained for 5 min before rapid cuff deflation. Recording of real-time duplex imaging was resumed 10 s before deflation and continued for ~3 min and ~6 min post-deflation, capturing the transient changes in brachial and popliteal artery flow and diameter of over a total of 200 and 350 cardiac cycles, respectively (Figure 2.2A).

Dilatory capacity (DC)

DC was assessed during the same measurement session after a rest period of 15 min. Conduit artery diameter and flow were re-measured and a return to baseline values confirmed before commencing the DC protocol. For the ischemic exercise DC stimulus, the pneumatic cuff was positioned upstream from the ultrasound probe (~10cm proximal to the olecranon process / popliteal fossa). The cuff was inflated > 200mmHg with occlusion maintained for 5 min. During the middle 3 min, rhythmic ischemic handgrip/plantar flexion exercise was performed using a dynamometer / stretch band at 20 contractions per minute (duty cycle of 1.5s concentric and 1.5s eccentric muscle actions). Real-time duplex imaging was performed as described for FMD (Figure 2.2B).

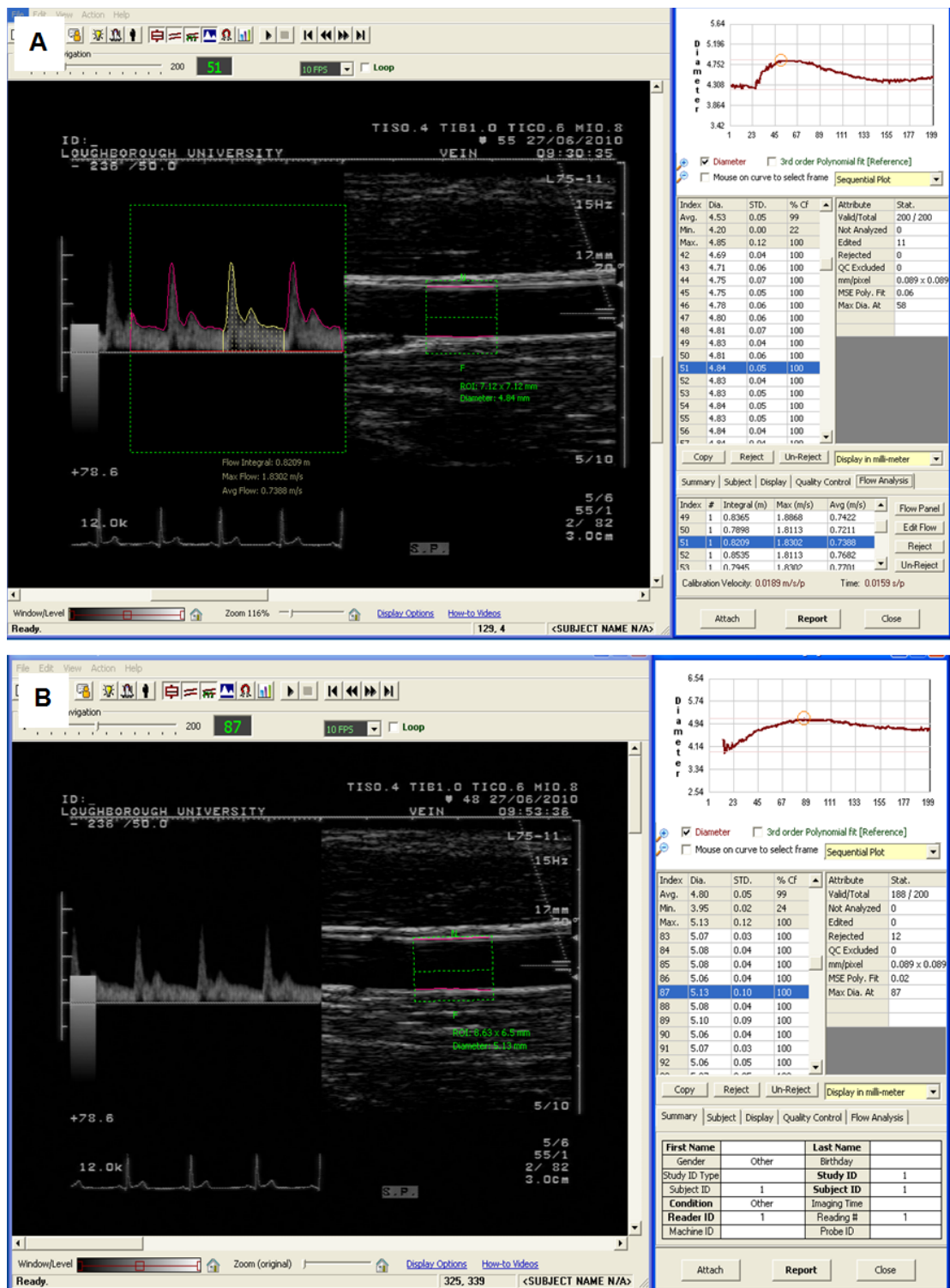


Figure 2.2 Duplex ultrasound display capturing brachial artery FMD (A) and DC (B). Peak (FMD) and maximal diameter (DC) occur at ~48s and ~77s post forearm and upper arm cuff release, respectively.

Ultrasound Data Analysis

Conduit artery diameter and flow velocity were analysed with a custom-designed, edge detection and wall tracking software (Vascular Research Tools 5, Medical Imaging Applications, LLC, Coralville, Iowa). Media-to-media diastolic diameter was measured within a specified region of interest on B-mode images. The Doppler flow velocity spectrum was traced and time average mean velocity (TAMV) ($\text{cm}\cdot\text{s}^{-1}$) computed. Synchronised diameter and velocity data, sampled at 20 Hz, enabled calculation of blood flow and shear rate. Resting diastolic diameter (mm) was averaged over 30 cardiac cycles. The dilatory response to FMD and DC protocols was determined from smoothed data (moving average across 3 cardiac cycles) and peak and maximal diameter defined, respectively. FMD and DC are presented as the absolute (mm) and relative (%) change in post-stimulus diameter [(maximum post stimulus diameter – baseline diameter)/baseline diameter]. Time to peak diameter (s) was calculated from the point of cuff deflation to the maximum post-deflation diameter. Blood flow ($\text{ml}\cdot\text{min}^{-1}$) was calculated as $(\text{TAMV} \times \pi r^2) \times 60$, where r is the radius of the brachial artery lumen. Resting blood flow was averaged over 30 cardiac cycles. Peak blood flow was defined as the highest area under the blood flow curve across a 10 s period following cuff deflation (Naylor *et al.*, 2005). Shear rate was derived from Poiseuillies law and calculated accordingly as $(4 \times \text{TAMV}) / \text{diameter}$. The accumulated shear stimulus contributing to the FMD response was defined as the area under the shear rate curve (SR_{AUC}) calculated for data up to the point of peak dilation for each individual (Figure 2.3). Given the uncertainty regarding an appropriate strategy for normalization of the FMD response, FMD% and SR_{AUC} were presented independently. For the legitimate application of normalised FMD in the present investigation, FMD% and SR_{AUC} should correlate (Y-intercept = 0) with data distributed normally (Harris *et al.*, 2010).

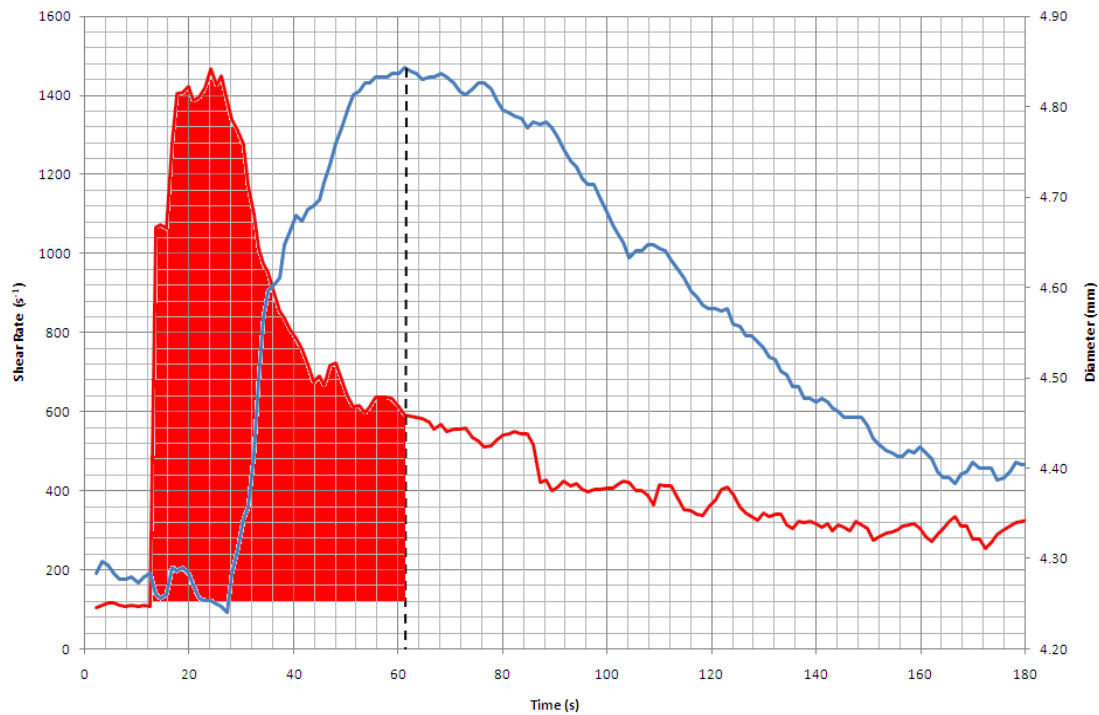


Figure 2.3 Schematic diagram of the area under the shear rate curve (SR_{AUC}) calculated for data up to the point of peak dilation.

Reproducibility and repeatability of Doppler ultrasound measures

The day-to-day variability of vascular measures derived from Doppler ultrasound was determined (Table 2.1 & 2.2). A student's paired t-test was performed for determination of statistical difference ($P > 0.05$) followed by a correlation coefficient to quantify the strength of the linear association between repeated measures. Bland-Altman method was used to measure agreement with repeated measures, with bias and 95% limits of agreement calculated.

Table 2.1 Brachial artery characteristics obtained from repeated measures ($n=9$)

Variable	Measure 1 \pm SD	Measure 2 \pm SD	r	CV	Mean Bias
FMD					
Baseline diameter (mm)	4.31 \pm 0.34	4.30 \pm 0.34	0.999†	0.2%	0.01 \pm 0.03
Peak diameter (mm)	4.63 \pm 0.34	4.62 \pm 0.35	0.992†	0.5%	0.01 \pm 0.09
FMD (mm)	0.32 \pm 0.11	0.32 \pm 0.11	0.951†	6.5%	0.00 \pm 0.07
FMD (%)	7.5 \pm 2.7	7.5 \pm 2.6	0.954†	6.4%	0.03 \pm 1.6
FMD (normalised, 10 ⁻⁵)	0.29 \pm 0.09	0.28 \pm 0.11	0.960†	5.7%	-0.01 \pm 0.10
Baseline velocity (cm s ⁻¹)	20.9 \pm 4.7	20.3 \pm 6.5	0.871*	16.0%	0.1 \pm 12.0
Baseline flow (ml min ⁻¹)	186 \pm 57	182 \pm 85	0.873*	15.8%	-4 \pm 93
Baseline SR (s ⁻¹)	195 \pm 49	185 \pm 46	0.779^	15.7%	6 \pm 117
Peak velocity (cm s ⁻¹)	110.7 \pm 26.2	108.3 \pm 24.4	0.920*	5.9%	4.3 \pm 20.4
Peak Flow (ml min ⁻¹)	971 \pm 236	921 \pm 277	0.922*	8.7%	55 \pm 239
Peak SR (s ⁻¹)	1032 \pm 276	1021 \pm 233	0.958†	5.4%	37 \pm 163
SR _{AUC} (10 ³ , s ⁻¹)	27.9 \pm 10.1	27.4 \pm 9.3	0.897*	7.9%	10.3 \pm 8.9
DC					
Baseline diameter (mm)	4.38 \pm 0.28	4.38 \pm 0.28	0.998†	0.2%	-0.00 \pm 0.04
Maximal diameter (mm)	4.94 \pm 0.24	4.93 \pm 0.24	0.990†	0.4%	0.00 \pm 0.06
DC (mm)	0.56 \pm 0.11	0.55 \pm 0.12	0.978†	3.1%	0.01 \pm 0.05
DC (%)	12.9 \pm 2.8	12.8 \pm 3.0	0.979†	3.1%	0.2 \pm 1.2
Baseline velocity (cm s ⁻¹)	17.9 \pm 4.9	15.7 \pm 3.7	0.733^	12.1%	0.5 \pm 11.1
Baseline flow (ml min ⁻¹)	156 \pm 54	137 \pm 48	0.856*	12.8%	2 \pm 102
Baseline SR (s ⁻¹)	169 \pm 46	146 \pm 30	0.683	12.4%	8 \pm 104

Significant ^ $P < 0.05$, * $P < 0.01$, † $P < 0.001$. FMD, flow mediated dilation; SR, shear rate; SRAUC, shear rate area under curve; DC, dilatatory capacity; r, correlation coefficients; SD, standard deviation; CV, coefficients of variation; Mean Bias \pm 95% limits of agreement

Table 2.2 Popliteal artery characteristics obtained from repeated measures ($n=9$)

Variable	Measure 1 \pm SD	Measure 2 \pm SD	r	CV	Mean Bias
FMD					
Baseline diameter (mm)	5.39 \pm 0.64	5.38 \pm 0.63	1.00†	0.5%	0.01 \pm 0.09
Peak diameter (mm)	5.64 \pm 0.61	5.66 \pm 0.61	0.99†	0.9%	-0.02 \pm 0.18
FMD (mm)	0.25 \pm 0.11	0.28 \pm 0.11	0.82*	17.4%	-0.03 \pm 0.13
FMD (%)	4.8 \pm 2.2	5.2 \pm 2.3	0.86*	17.2%	0.01 \pm 2.37
FMD (normalised, 10 ⁻⁵)	18.9 \pm 8.9	20.4 \pm 12.9	0.63	25.9%	-2.7 \pm 21.3
Baseline velocity (cm s ⁻¹)	19.2 \pm 8.2	19.7 \pm 7.8	0.79^	16.3%	-0.46 \pm 10.2
Baseline flow (ml min ⁻¹)	270 \pm 137	275 \pm 122	0.80^	17.5%	-5.0 \pm 161.7
Baseline SR (s ⁻¹)	144.5 \pm 61.5	145.5 \pm 54.8	0.77^	16.0%	-0.9 \pm 77.6
Peak velocity (cm s ⁻¹)	113.6 \pm 50.1	116.7 \pm 30.4	0.84*	14.8%	-3.1 \pm 54.8
Peak Flow (ml min ⁻¹)	1496 \pm 723	1586 \pm 538	0.86*	14.2%	90 \pm 745
Peak SR (s ⁻¹)	872 \pm 387	884 \pm 222	0.87*	15.8%	-12 \pm 437
SR _{AUC} (10 ³ , s ⁻¹)	27.5 \pm 15.6	30.4 \pm 15.8	0.87*	19.2%	-2.9 \pm 1.6
DC					
Baseline diameter (mm)	5.31 \pm 0.64	5.35 \pm 0.70	0.99†	0.9%	-0.03 \pm 0.21
Maximal diameter (mm)	5.88 \pm 0.65	5.90 \pm 0.70	1.00†	0.7%	-0.01 \pm 0.13
DC (mm)	0.52 \pm 0.11	0.48 \pm 0.13	0.89^	9.1%	0.03 \pm 0.11
DC (%)	9.8 \pm 2.9	9.1 \pm 3.1	0.92*	9.8%	0.6 \pm 2.2
Peak velocity (cm s ⁻¹)	137.8 \pm 47.3	133.7 \pm 49.8	0.99†	3.8%	3.1 \pm 11.1
Peak flow (ml min ⁻¹)	1625 \pm 771	1604 \pm 811	0.99†	5.8%	16 \pm 189
Peak SR (s ⁻¹)	1130 \pm 327	1108 \pm 346	0.98†	4.1%	17 \pm 112

Significant, ^ $P < 0.05$, * $P < 0.01$, † $P < 0.001$. FMD, flow mediated dilation; SR, shear rate; SRAUC, shear rate area under curve; DC, dilatatory capacity; r , correlation coefficients; SD, standard deviation; CV, coefficients of variation; Mean Bias \pm 95% limits of agreement.

Brachial artery measures: Intra-observer coefficient of variation for repeated measures of FMD was 6.4%, which is comparable to previous investigations (6.7-10.5%, Tinken *et al.*, 2010; 3.4%, Peretz *et al.*, 2007; 14.7%, Woodman *et al.*, 2001). Ischemic exercise evokes a maximal dilatory stimulus, diminishing the impact of variability in vascular function and explaining the reduced CV% in DC (3.1%).

Popliteal artery measures: There was no correlation between repeated measures of normalised FMD due largely to variance in the SR_{AUC} response. Intra-observer coefficient of variation for repeated measures in FMD was 17.4%, which compares favourably to previous investigations of the popliteal artery (28%, Rakobowchuk *et al.*, 2008). The popliteal artery maximal dilatory response to ischemic exercise demonstrated greater reproducibility (9.8%) in agreement with brachial artery observations.

Blood velocity measures in the brachial and popliteal artery are consistently higher than previously reported. This is likely due to the central positioning of the Doppler sample volume within the artery and minimised gate width, which given the parabolic flow profile, heightened the velocity spectrum and TAMV. Subsequently calculations of shear rate and blood flow were augmented. Absolute values may therefore conflict with others in the literature but given the consistent placement and size of the sample volume (gate width) with repeated measures this has no impact on the research findings.

2.3.2 Strain gauge plethysmography

Strain-gauge plethysmography measures were carried out in the supine position. A mercury strain gauge was placed around the widest portion of the limb and attached to a dual channel plethysmograph (EC6 Plethysmograph, Hokanson, Bellvue, WA, USA). The strain gauge was used to detect volume changes in the limb in response to proximal pneumatic cuff (Hokanson, Bellvue, WA, USA) inflation (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA, USA). Strain gauge signals were sampled on line at 100Hz (Powerlab, AD Instruments, NSW, Australia) and the slope of the volume change measured using Chart version 5 software (ADInstruments, NSW, Australia).

Forearm blood flow

The forearm was supported in line with the heart using a 15 cm and 7 cm foam block under the elbow and hand. The strain gauge was placed around the widest circumference of the forearm. Pneumatic cuffs (Hokanson, Bellvue, WA, USA) were positioned 2-3 cm proximal to the olecranon process and around the wrist.

Resting forearm blood flow; Thirty seconds prior to blood flow measurements arterial blood flow to the hand was occluded by inflating the wrist cuff to 300 mmHg. After 45 s, blood flow was measured by inflating the proximal cuff to a venous occlusion pressure of 50 mmHg for ~7 s after which the cuff was deflated. Blood flow was calculated from the slope of the volume change over the first cardiac cycle (Tschakovsky *et al.*, 1995) following cuff inflation and expressed in $\text{ml}\cdot\text{min}^{-1}\cdot 100\text{ml}^{-1}$ of tissue (Figure 2.4). This inflation-deflation process was repeated 3 times, with approximately 30 s between each measurement, and the average taken. The wrist cuff was deflated immediately after the final blood flow measurement was obtained.

Post-occlusion blood flow Following measurement of resting flow, the reactive hyperaemic flow response to forearm ischemia was assessed while the participant remained in the supine position. The proximal cuff was inflated to 200 mmHg for 5 min. With 45 s of occlusion remaining, the wrist cuff was inflated to 300 mmHg. Following rapid deflation, measurements of forearm blood flow were obtained at 10, 20 & 30 s and every 15 s thereafter for 2 min. Peak post-occlusion blood flow was defined as the highest blood flow value after occlusion.

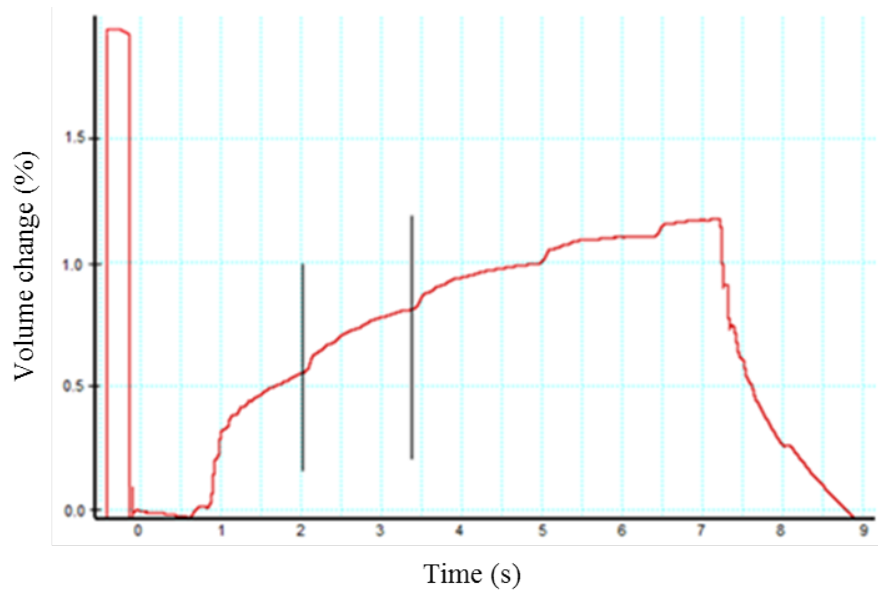


Figure 2.4 Strain gauge forearm volume profile immediately preceding, during and after occlusion cuff inflation to 50mmHg. Vertical solid lines 1st beat calculation of forearm blood flow. Vertical dashed lines cuff inflation and deflation.

Calf filtration capacity

The calf was supported in line with the heart using a 7cm and 15cm foam block under the knee and ankle. The strain gauge was placed around the widest circumference of the calf and a pneumatic cuff (Hokanson, Bellvue, WA, USA) positioned at the mid thigh level. After 20 min supine rest, arterial blood pressure was recorded at the brachial artery using an automated sphygmomanometer (Omron M5-1 Digital BP monitor, Omron Healthcare, The Netherlands). A series of eight cumulative pressure increments were applied to the thigh cuff without exceeding diastolic pressure. Each pressure step of ~7-9 mmHg was maintained for 4 min. In response to each pressure increment (which exceeded isovolumetric venous pressure) there was a two phase calf volume response; 1) the initial rapid phase attributed to venous filling (complete within 90 s) and 2) a subsequent slower phase attributed to fluid filtration (Gamble *et al.*, 1993) (Figure 2.5A). The fluid filtration rate (J_v) was measured from the slope of volume change during the 2-4th min of each pressure step. This filtration rate was plotted against cuff pressure, and the slope of the linear relationship defined the filtration capacity (K_f ; $\text{ml} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1} \cdot 100\text{ml tissue}^{-1} \times 10^{-3}$). This linear relationship was extrapolated to determine the isovolumetric venous pressure (P_{vi} ; mmHg) at the x-axis (cuff pressure) intercept (Figure 2.5B).

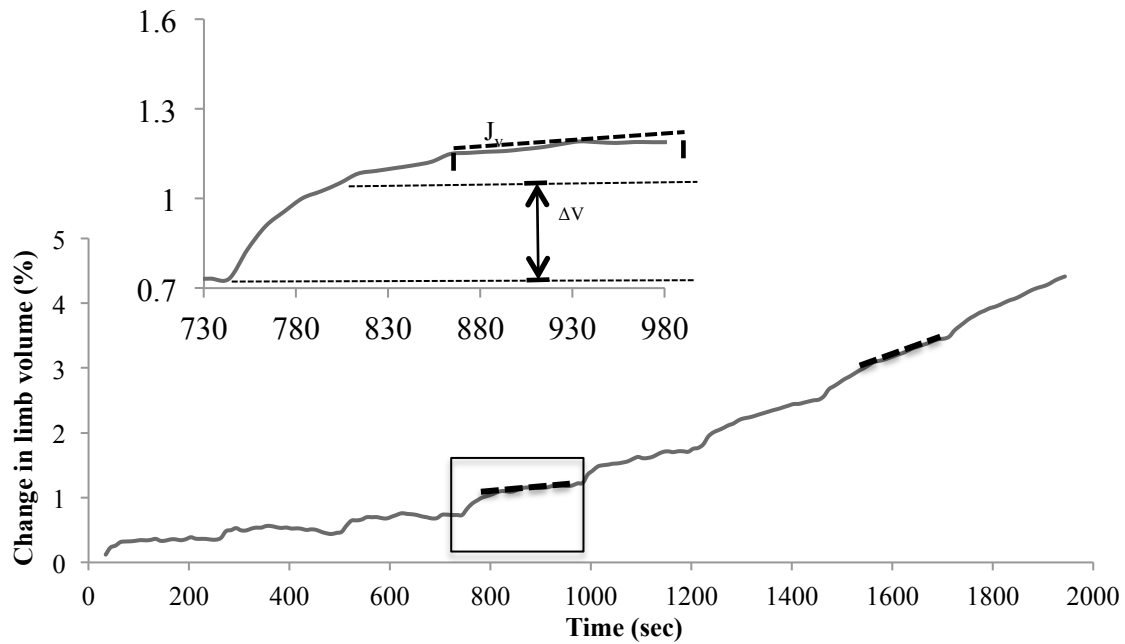
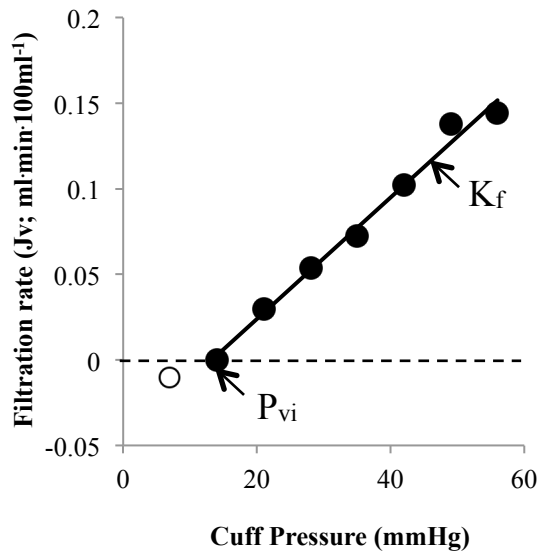
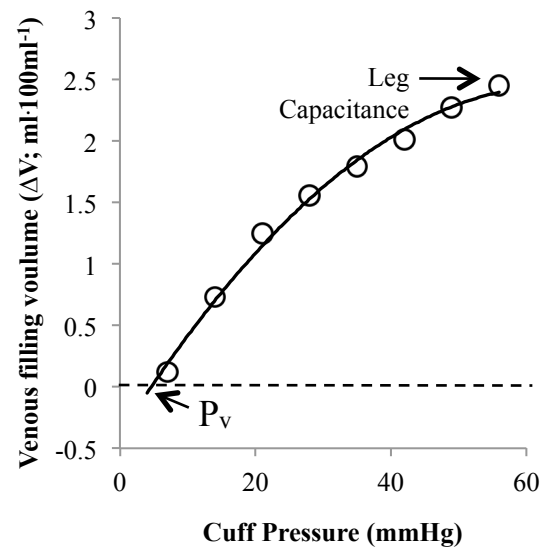
A**B****C**

Figure 2.5 (A) A plethysmographic trace depicting the change in calf volume in response to eight cumulative pressure increments; the stimulated response to a single cuff pressure step is magnified and partitioned into contributions from capacitance vessel filling (ΔV) and from microvascular fluid filtration (J_v). The relationship between the values of cuff pressure and corresponding filtration rate (B), and vascular filling volume (C). The intercept, indicated by an arrow, gives the isovolumetric venous pressure (P_{vi}) and venous pressure (P_v).

The residual volume change due to venous filling (capacitance vessel filling) was determined by stripping the filtration slope from the total strain gauge response to each pressure increment. The curvilinear relationship between vascular filling volume and cuff pressure was plotted (Figure 2.5C), representing a function of the vascular and surrounding tissue compliance (Gamble *et al.*, 1997). Venous pressure (P_v ; mmHg) was estimated at the x-axis (cuff pressure) intercept by extrapolating the relationship using a curvilinear fitting routine (polynomial 2nd order) (Christ *et al.*, 1997). Leg capacitance was calculated from the sum of the residual portions attributable to venous filling and expressed as normalised volume in units of ml volume change/100 ml tissue.

2.4 Muscle biopsy measures

2.4.1 Muscle biopsy sampling

Muscle biopsies were conducted by the same individual who had extensive experience in performing the procedure. Samples (~ 30 mg wet wt) were obtained, from the vastus lateralis at a location distal to the occlusion site (cuff location during BFR exercise), under local anesthesia (1% lidocaine), using the needle micro-biopsy technique (Acecut 11G Biopsy Needle, TSK). Two passes were made, with samples placed into separate tubes (one containing 500µl TRIzol reagent) before being immediately frozen in liquid nitrogen and stored at -80°C until analysis. Biopsies were alternated between legs and sites were separated by at least 3 cm as previously described (Gavin *et al.*, 2004). The resting biopsy sample was counterbalanced to the dominant and non-dominant leg.

2.4.2 RNA isolation

Muscle samples (~15-20 mg) snap frozen in 500µl TRIzol reagent (Sigma-Aldrich 93289) for mRNA analysis, were briefly thawed before being gently homogenised, over ice, using a handheld rotor-stator TissueRuptor probe (QIAGEN). The supernatant was transferred to a new tube (RNase free) and left at room temperature for 5 min. Following the addition of 100µl chloroform, the sample was vigorously shaken for 15 s and incubated at room temperature for a further 2-3 min. Centrifugation (12000 x g for 15-min at 4°C) separated the

solution into 3 phases: a red organic phase (containing protein), an interphase (containing cDNA) and a colourless aqueous phase (containing RNA). To isolate RNA, the upper aqueous phase was transferred to a new tube (RNase free) and mixed with 250µl of isopropyl alcohol. The sample was incubated at room temperature for 10 min before centrifugation at 12000 x g for 10 min at 4°C. The RNA precipitate formed a pellet on the side/bottom of the tube. The supernatant was discarded and the RNA pellet washed with 75% ethanol before 5 min incubation at room temperature and centrifugation (7500 x g for 5 min at 4°C). The supernatant was removed and RNA pellet air dried for 15-min. The RNA was re-suspended in 50µl of RNA storage solution, aliquoted into multiple tubes and stored at -80 for later analysis. The RNA concentration and purity was determined by spectrophotometry (Nanodrop). Total RNA was quantified by absorbance at 260nm. The purity of samples was assessed from the ratio of absorbance at 260 and 280 nm, which was always between 1.7 and 2.1.

2.4.3 Real-time qualitative PCR

Real-time RT-PCR was used to measure VEGF, VEGF-R2, PGC-1 α , HIF-1 α , eNOS, MMP-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression. The real time RT-PCR reactions were carried out using QuantiFast SYBR Green one step RT-PCR Kit (QIAGEN) with runs performed on an Mx3000P/Mx3005P QPCR cyclers (Stratagene, Agilent Technologies). The QuantiFast SYBR Green RT-PCR Kit contained a 2x QuantiFast SYBR Green RT-PCR master mix (Taq polymerases, SYBR Green buffer, dNTP mix and fluorescence dyes [SYBR Green 1 & ROX]) together with a Quantifast RT mix, enabling reverse transcription and PCR in a single tube setup. One step RT-PCR was performed in duplicate, with 70ng of RNA, in a total reaction volume of 20µl. The 20 µl reaction mix was comprised of 0.2µl reverse transcriptase (QuantiFast RT mix), 10µl of SYBR Green master mix (QuantiFast, QIAGEN), 0.15µl of each forward and reverse PCR primers and 9.5µl RNA. Primer sequences were designed by SIMGA (Table 2.3)

Table 2.3 DNA oligonucleotide primers used for real time PCR

Primer description	Sequence (5'-3')
VEGF Forward	ATGAACTTCACCACTTCGT
VEGF Reverse	CTGCTCTACCTCCACCAT
VEGFR-2 Forward	TTACTTCTGGTTCTTCTAACG
VEGFR-2 Reverse	AGCAGGAATCAGTCAGTAT
PGC-1 α Forward	ACTCTCGCTTCTCATACTC
PGC-1 α Reverse	CCTCTTCAAGATCCTGCTA
HIF-1 α Forward	AATCTGTGTCCTGAGTAGAA
HIF-1 α Reverse	TCACCTGAGCCTAATAGTC
eNOS Forward	TGTGAAGGCTGTAGGTTAT
eNOS Reverse	CAAGTTGGAATCTCGTGAA
MMP-9 Forward	GGCACCTCTATGGTCCTC
MMP-9 Reverse	AGTAGTGGCCGTAGAAGG
GAPDH Forward	GGTGGAAATCATATTGGAACAT
GAPDH Reverse	GAGTCAACGGATTTGGTC

An identical RT-PCR cycle profile was used for all genes. Real-Time PCR was run for 1 cycle (50°C for 10 min [RT], 95°C for 5min [PCR initial activation step]) immediately followed by a two-step cycling protocol (95°C for 10s [denaturation], 60°C for 30s [combined annealing/extension step]) for 30-40 cycles. Fluorescence was measured after each of the repeated cycles. On completion of PCR, all PCR products formed were melted to attain a melting curve profile, which enabled the specificity of the reaction to be determined. GAPDH was used as the housekeeping gene as it remained stable across time for both trials.

Results were expressed using the comparative cycle threshold (Ct) method. The Ct represents the PCR cycle at which an increase in reporter gene fluorescence above a baseline signal can be detected. Post exercise values were reported as a fold difference relative to the expression of the pre-exercise values. Relative fold changes were determined using the $2^{-\Delta\Delta C_t}$ method using the equation: Fold = $2^{(C_t \text{ target gene} - C_t \text{ housekeeping gene}) \text{ time x} - (C_t \text{ mean target gene} - C_t \text{ mean housekeeping gene}) \text{ baseline}}$.

2.5 Muscle strength measures

One repetition maximum (1RM) was determined for handgrip (chapter 3), plantar flexion (chapter 4) and knee extension (chapter 5) exercise on training specific dynamometers (Figure 2.6). The load was set at 80% of the predicted 1RM and increased by 5% with each successful attempt. A successful lift was acknowledged when the participant completed the repetition through the full range of motion at the maximum weight possible. The 1-RM was achieved within 5 attempts each separated by 4 min of rest.

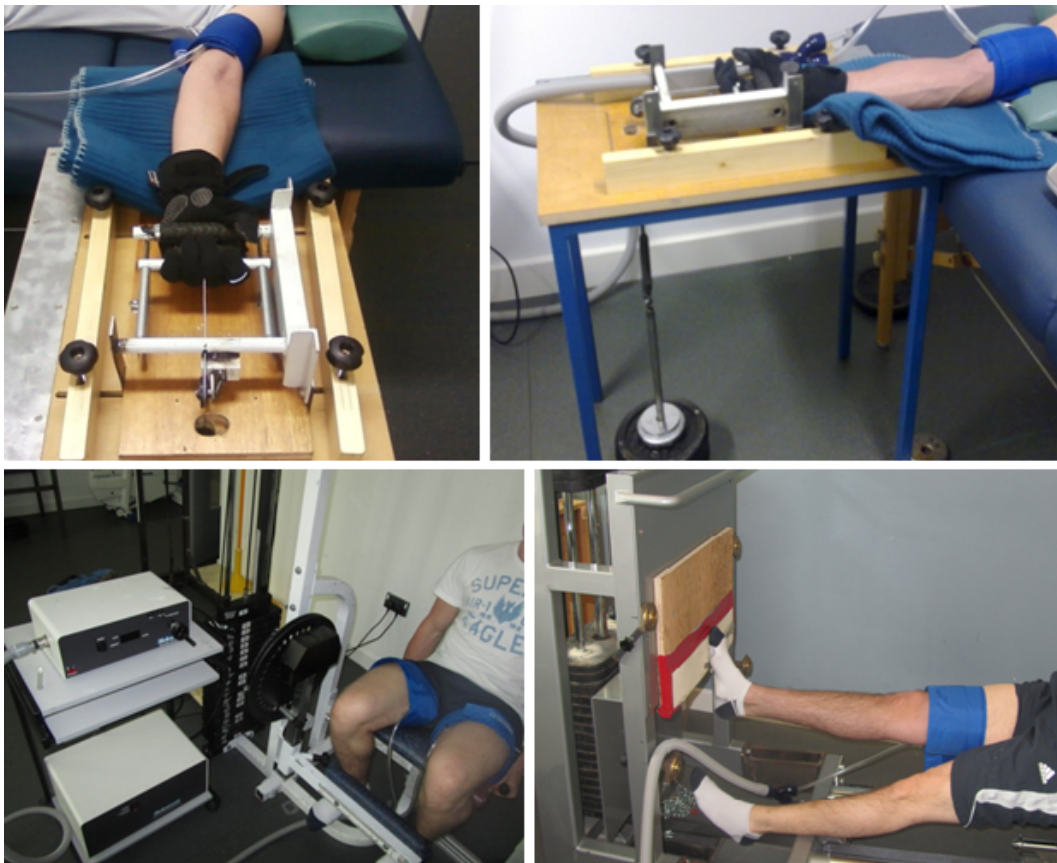


Figure 2.6 Handgrip, knee extension and plantar flexion dynamometers.

2.6 Anthropometric measures

In training studies (chapter 3-4) the widest circumference of the experimental and control limb was measured as a marker of muscle hypertrophy. In chapter 6, limb circumference of

the thigh (mid-point between the inguinal fold and the anterior aspect of the patella) and upper arm (mid-acroniale-radiale distance) was determined, reflecting the site of cuff application.

In chapter 6, subcutaneous adipose tissue thickness overlying the quadriceps, hamstrings, biceps, and triceps were measured using B-mode ultrasound (Toshiba Powervision 6000) with a linear array transducer (12MHz). Measures were made at circumferential marks (see above) to capture an accurate representation of the site at which cuffs would be applied.

Orientated in the sagittal-plane, the centre of the transducer was aligned perpendicular to the horizontal circumferential line, on the midline of the anterior and posterior surface of the limb. Subcutaneous adipose tissue was imaged at depth of 2-5cm, depending on the site and individual. Minimal force was applied through the transducer to avoid compression of the adipose layer. Two ultrasound B-mode images were systematically acquired at each site (quadriceps, hamstrings, biceps and triceps) using vascular imaging software (Medical Imaging Applications, LLC, Coralville, Iowa) and later analysed using Image J (Figure 2.7). On each image, subcutaneous fat thickness was measured at four sites and the average of eight values recorded.

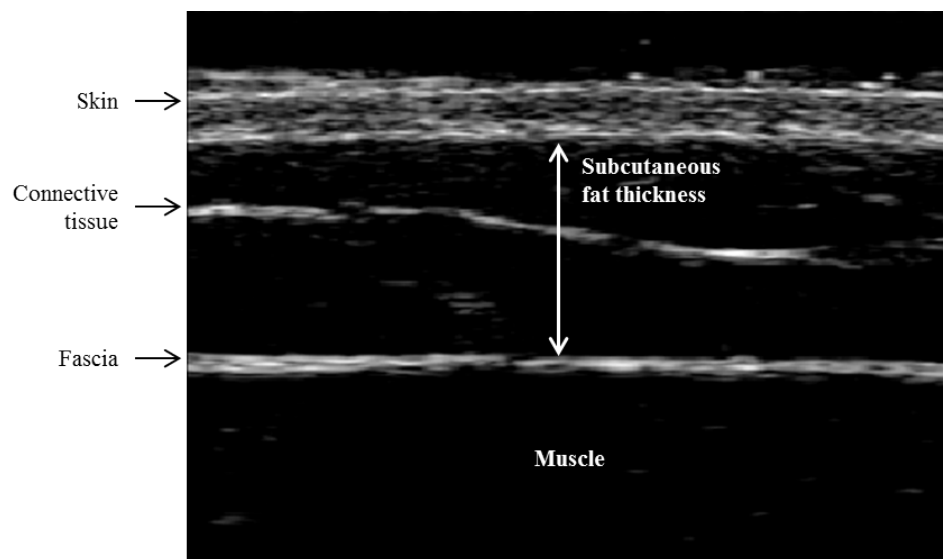


Figure 2.7 B-mode ultrasound image of the subcutaneous adipose tissue overlying the quadriceps

2.7 Exercise protocols

In chapters 3-5 protocols involved dynamic exercise with concentric-eccentric muscle actions (the concentric ‘lifting’ phase followed by the eccentric ‘lowering’ phase). Unilateral limb exercise (engaging a small muscle group) was used for longitudinal training investigations (chapters 3, 4) to assess the impact of localised muscle activity on regional vascular adaptations, in the absence of central hemodynamic activation. Handgrip exercise is traditionally conducted alongside conventional measures of forearm hyperaemia and brachial function to evaluate resistance and conduit vessel training adaptation (Green *et al.*, 1994). Subsequently, the first study (chapter 3) used handgrip exercise to assess locally mediated vascular adaptations in response to training with (experimental arm) and without BFR (contralateral control arm). In chapter 4, lower limb training was performed in keeping with the majority of BFR exercise models. Supine plantar flexion exercise was used to assess regional vascular (conduit, resistance and capillary vessels) changes in the BFR trained leg compared to the non-exercise control leg. Bilateral knee extension exercise was used to assess the systemic and local response to acute resistance exercise with and without BFR (chapter 5).

Training load was set at 40%, 30% and 20% 1RM in chapters 3-5 respectively. In each case repetitions were performed to concentric failure at a frequency of 20 contractions per minute (duty cycle of 1.5s concentric and 1.5s eccentric muscle actions). The intensity of the low load training was manipulated for each exercise mode to ensure a sufficient total volume of work (60+ repetitions) was performed within a limited duration (<10min) under BFR conditions. These variables are believed to be important for the optimisation of low load BFR training (Fahs *et al.*, 2012b).

2.8 Blood flow restriction

Upper arm and thigh segmental pressure cuffs (Hokanson, Bellvue, WA, USA) were used to implement venous occlusion during BFR exercise. Pressure in the pneumatic cuff was controlled by a Rapid Cuff Inflator and a Cuff Inflator Air Source (E20, AG101, Hokanson,

WA, USA). Inflation pressure was set according to the restrictive cuff width, limb size and exercise mode to elicit venous occlusion and reduce arterial inflow, while ensuring the necessary exercise tolerance for the target workload (Yasuda *et al.*, 2008; Fahs *et al.*, 2012b). To determine the BFR stimulus, the absolute and relative level of restricted blood flow was measured at different cuff pressures applied on the upper thigh (90-150mmHg) and arm (60-120mmHg) in chapter 6.

For BFR handgrip training (chapter 3) an 11 cm cuff (SC10TM segmental pressure cuff, Hokanson, WA) was applied around the upper arm and inflated to 80 mmHg. Previous studies have utilised similar restrictive cuff pressures during isometric handgrip exercise (Credeur *et al.*, 2010). Restriction of venous outflow occurs at low pressures (7-35mmHg) in a supine position (Ludbrook & Collins, 1967; Fahs *et al.*, 2012), causing pooling in the forearm vasculature distal to the cuff. Small muscle mass recruitment, given the exercise mode and intensity, minimised venous outflow induced by the skeletal muscle pump. Arterial inflow is also reduced as a result of vasoconstriction, an additive response to the pressure exerted by the cuff and the active tone of the brachial artery (Hiatt *et al.*, 1989).

For BFR planter flexion training (chapter 4) and knee extension (KE) exercise (chapter 5) a 13 cm cuff (SC12LTM segmental pressure cuff, Hokanson, WA) was applied around the thigh and inflated to 110mmHg. This restrictive cuff pressure was utilised by Paterson & Ferguson (2010) in a planter flexion training program which enhanced strength and blood flow capacity. Cole & Brown (2000) have previously demonstrated that inflation of a thigh cuff (14 cm width) to 100mmHg reduces ankle-brachial systolic pressure index to 0.8. This level of partial ischemia in the lower leg matches that experienced by patients with mild peripheral artery disease (ankle-brachial systolic pressure index 0.51-0.95).

2.9 Statistical analysis

For all investigations a Shapiro-Wilk test was used to confirm normal distribution and a Mauchley test of sphericity to verify homogeneity of variance. In chapters 3-5, initially a two way ANOVA with repeated measures was conducted to analyse the within-subject effect of exercise condition (blood flow restricted, control) and time. This was followed by one-way repeated measures ANOVA to confirm change over time in each limb (chapters 3, 4) or trial

(chapter 5) separately. Bonferroni post hoc t-tests were then used to locate significance. Chapter 6 used one-way repeated measures ANOVA to confirm change in artery diameter and blood flow with incremental levels of restrictive cuff pressure. Following this, models of hierarchical linear regression were used to determine pressure at which 60% blood flow restriction occurred. Pearson's correlation, adjusted R^2 , standard error of the estimate (SEE) and the change in F value were reported at each level of the regression model. All data are presented mean \pm SD, unless otherwise stated. The level of significance for all statistical analysis was accepted at $P < 0.05$.

Chapter 3: Peripheral vascular modifications to blood flow restricted handgrip training and detraining

3.1 Introduction

Low load resistance training with BFR has been shown to elicit similar hypertrophic and strength gains as traditional high load resistance training (Manini & Clark, 2009). The alteration in normal blood flow, on which this modality is based, exposes the vasculature to distorted hemodynamic (Takano *et al.*, 2005), redox (Kawada, 2005; Clanton, 2007) and chemical/metabolic signals (Takano *et al.*, 2005; Reeves *et al.*, 2006; Suga *et al.*, 2009), which could all have adaptive effects on the vasculature. However, relatively little is known regarding the effect of BFR training on the peripheral vasculature.

Conduit artery modifications have been shown to occur in response to localised short-term resistance exercise training (Allen *et al.*, 2003; Tinken *et al.*, 2010; Thijssen *et al.*, 2011b). A transient increase in FMD is often evident before structural remodelling of the conduit artery (Tinken *et al.*, 2008, 2010). Although primarily a measure of conduit endothelial function, the FMD response is largely influenced by the magnitude of the reactive hyperaemic shear stimulus, governed by the extent of ischemic micro-vascular dilation (Malik *et al.*, 2004). Since low load resistance exercise training with BFR can increase reactive hyperaemic blood flow (reflecting resistance vessel dilatory capacity) (Patterson & Ferguson, 2010) and micro-vascular filtration capacity (reflecting capillarity) (Evans *et al.*, 2010) an enhanced FMD response would be expected. Intriguingly, both acute (Renzi *et al.*, 2010) and chronic (Credeur *et al.*, 2010) BFR exercise have been shown to decrease conduit artery FMD. It is assumed that this reduced FMD is a product of diminished endothelial function; however, alterations to conduit artery geometry could also explain findings. Larger arteries display a smaller dilatory response to functional stimulation (Schroeder *et al.*, 2000; Thijssen *et al.*, 2008) raising the possibility that structural enlargement of the conduit artery could account for decreased FMD. Maximal dilation after ischemic exercise is an accepted (Naylor *et al.*, 2005) and frequently used (Tinken *et al.*, 2008, 2010) method to examine conduit structure. This technique could be used, alongside FMD measures, to verify whether the alterations in artery function occur as a result of structural changes following BFR training.

Changes to conduit artery structure and function after removal of the BFR training stimulus have not been investigated. Several experimental models demonstrate that inactivity (e.g. deconditioning or limb unloading) provides a strong stimulus for rapid structural remodelling of the conduit arteries and changes to reactive hyperaemic flow (Bleeker *et al.*, 2005; Rakobowchuk *et al.*, 2011). Therefore, a detraining effect will identify the vascular response to changes within the local milieu caused by the removal of contraction and BFR induced shear, transmural and metabolic stress.

Therefore, the aim of the present investigation was to examine the effects of low load dynamic handgrip training with and without BFR, and detraining, on measures of brachial artery function, structure and forearm reactive hyperaemia.

3.2 Methods

3.2.1 Participants

Nine healthy males (age; 26 ± 4 yrs, height; 178.1 ± 3.2 cm, body mass; 78.1 ± 9.5 kg, resting systolic blood pressure: 127 ± 6 mmHg, resting diastolic blood pressure: 70 ± 7 mmHg) volunteered to take part in the investigation. All participants were habitually physically active but none were partaking in resistance exercise training. The participants were fully informed of the purposes, risks and discomforts associated with the experiment before providing written, informed consent. This study conformed to current local guidelines and the Declaration of Helsinki and was approved by Loughborough University Ethics Advisory Committee.

3.2.2 Experimental design

Participants completed four weeks (3 sessions / week) of unilateral dynamic handgrip training at 40% 1 repetition maximum (1RM) to volitional fatigue under restricted and non-restricted blood flow conditions. This was followed by two weeks of no training (de-training). Measures of brachial artery function (FMD) and structure (maximum diameter), forearm resting and post occlusion blood flow, and handgrip strength (1RM) were made on both arms, before (pre-training), after training (post-training) and after de-training. Post training measures took place 2 days after the last exercise session.

3.2.3 Familiarization

Participants were familiarised with testing procedures and training devices during two preliminary visits, 1 week prior to experimental measures. During the first visit, brachial artery properties were assessed in the right arm in accordance with the experimental protocol (described below). This established optimal brachial artery image location and familiarised the participant with the occlusion procedures. During the second preliminary visit, participants were familiarised with the handgrip exercise device, 1RM was determined and a BFR handgrip training protocol experienced.

3.2.4 Experimental Protocol

Participants maintained a habitual pattern of physical activity and dietary habits during the study period. Brachial artery measures (pre-, post- and de-training) were conducted in both arms at the same time in the morning (± 1 h), following a 10 h overnight fast, and in a quiet temperature controlled room ($24 \pm 1^\circ\text{C}$). Participants abstained from vitamin supplementation for 72 h and from exercise, alcohol and tobacco for 24 h prior to vascular tests. Upon arrival participants lay supine for 20 min prior to basal assessment. Brachial artery properties were first assessed using Doppler ultrasound followed by blood flow measures using venous occlusion plethysmography. The right arm was always imaged first with a 15 min period allowed between each assessment. Following vascular assessments forearm anthropometry and strength measurements were then made.

3.2.5 Brachial artery assessments

Doppler ultrasound imaging was performed by the same sonographer using a Toshiba Powervision 6000 with a multi-frequency linear array transducer (7-11 MHz). Participants lay supine with imaged arm extended and immobilised at an angle approximately 80° from the torso. A 90° B-mode image of the brachial artery at 3 cm depth was obtained >3 cm proximal of the olecranon process. In duplex mode the sample volume was centralised within the artery and the ultrasound beam aligned with direction of flow (insonation angle $\leq 69^\circ$). Once a satisfactory image was made, the probe was held in a constant position for the duration of the test. Ultrasound settings were standardised for each individual and kept constant for repeated measures. A vascular ECG gating module (Medical Imaging

Applications) triggered acquisition of the ultrasound images on the R-wave pulse of an ECG signal. Sequential end-diastolic images were stored from on-line image digitization.

Flow mediated dilation (FMD) Resting brachial diameter and blood flow were recorded for 20 cardiac cycles following a 20 min rest period. For the ischemic FMD stimulus a pneumatic cuff (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA, USA) was placed immediately distal to the olecranon process and inflated to 200 mmHg. Occlusion was maintained for 5 min before rapid cuff deflation. Recording of real-time duplex imaging was resumed 10 s before deflation and continued for ~3 min post-deflation, capturing the transient changes in flow and diameter over a total of 200 cardiac cycles.

Dilatory capacity (DC) Brachial diameter and flow were re-measured and a return to baseline values ensured before commencing the DC protocol. For the ischemic exercise DC stimulus, the pneumatic cuff was positioned on the upper arm proximal to the ultrasound probe. The cuff was inflated to 200mmHg with occlusion maintained for 5 min. During the middle 3 min, rhythmic ischemic handgrip exercise was performed using a 10kg weight at 20 contractions/min. Real-time duplex imaging was performed as described for FMD.

Ultrasound Data Analysis Brachial artery diameter and flow velocity were analysed with a custom-designed, edge detection and wall tracking software (Vascular Research Tools 5, Medical Imaging Applications, LLC, Coralville, Iowa). Media-to-media diastolic diameter was measured within a specified region of interest on B-mode images. The Doppler flow velocity spectrum was traced and TAMV (cm/s) computed. Synchronised diameter and velocity data, sampled at 20 Hz, enabled calculation of blood flow and shear rate. Resting diastolic diameter (mm) was averaged over 20 cardiac cycles. The dilatory response to FMD and DC protocols was determined from smoothed data (moving average across 3 cardiac cycles) and peak and maximal diameter defined, respectively. FMD and DC are presented as the absolute (mm) and relative (%) change in post-stimulus diameter; [(maximum post stimulus diameter – baseline diameter)/baseline diameter]. Time to peak diameter (s) was calculated from the point of cuff deflation to the maximum post-deflation diameter. Blood flow (ml/min) was calculated as $(\text{TAMV} \times \pi r^2) \times 60$, where r is the radius of the brachial artery lumen. Resting blood flow was averaged over 20 cardiac cycles. Peak blood flow was recorded as the highest value (across a single cardiac cycle) following cuff deflation. Shear rate was derived from Poiseuillies law and calculated accordingly as $(4 \times \text{TAMV})/\text{diameter}$.

The accumulated shear stimulus contributing to the FMD response was defined as SR_{AUC} and calculated for data up to the point of peak dilation for each individual. Given the uncertainty regarding an appropriate strategy for normalization of the FMD response, FMD% and SR_{AUC} were presented independently. The day-to-day reproducibility of brachial artery measurements was; diameter (0.5%), blood flow (8.7%), FMD% (6.5%) and DC% (3.1%) respectively.

3.2.6 Peripheral vascular assessments

Forearm blood flow measurements were carried out using venous occlusion strain-gauge plethysmography (Hokanson, Bellvue, WA, USA). The forearm was supported in line with the heart using a 15 cm and 7 cm foam block under the elbow and hand. A mercury strain gauge was placed around the widest circumference of the forearm and attached to a dual channel plethysmograph (EC6 Plethysmograph, Hokanson, Bellvue, WA, USA). Strain gauge signals were sampled on line at 100 Hz (Powerlab, AD Instruments, NSW, Australia). Pneumatic cuffs (Hokanson, Bellvue, WA, USA) were positioned 2-3 cm proximal to the olecranon process and around the wrist. Rapid inflation of proximal cuff occurred by connection to a pneumatic air source (E20 Rapid cuff inflator and AG101 Cuff Inflator Air Source, Hokanson, WA, USA). Forearm blood flow was calculated from the slope of the volume change over the first cardiac cycle (Tschakovsky *et al.*, 1995), using Chart version 5 software (ADInstruments, NSW, Australia) and expressed in $ml/min/100ml$ of tissue.

Resting forearm blood flow Thirty seconds prior to blood flow measurements arterial blood flow to the hand was occluded by inflating the wrist cuff to 300 mmHg. After 45 s, blood flow was measured by inflating the proximal cuff to a venous occlusion pressure of 50 mmHg for ~7 s after which the cuff was deflated. This process was repeated 3 times, with approximately 30 s between each measurement, and the average taken. The wrist cuff was deflated immediately after the final blood flow measurement was obtained.

Post-occlusion blood flow Following measurement of resting flow, the reactive hyperaemic flow response to forearm ischemia was assessed while the participant remained in the supine position. The proximal cuff was inflated to 200 mmHg for 5 min. With 45 s of occlusion remaining, the wrist cuff was inflated to 300 mmHg. Following rapid deflation measurements

of forearm blood flow were obtained at 10, 20 & 30 s and every 15 s thereafter for 2 min. Peak post-occlusion blood flow was defined as the highest blood flow value after occlusion.

3.2.7 Forearm strength and anthropometry

1-RM was determined on a custom made handgrip dynamometer. Participants flexed their fingers which lifted and lowered weights (kg) hanging over a pulley. To isolate the finger flexors and extensors as the sole producers of force, participants lay supine with arm extended 90° at the shoulder and forearm in supination. A successful lift was acknowledged when the participant completed the repetition with a full range of movement (6-8 cm) at the maximum weight possible. The 1-RM was achieved within 5 attempts. Maximum forearm circumference was measured in supination at one-sixth of the distance between the olecranon process and ulnar styloid. Forearm volume was determined using water displacement; the arm was submerged to a proximal marker (olecranon process) and water discarded before immersion to a distal marker (ulnar styloid) and the volume of water displaced measured.

3.2.8 Exercise training

Participants completed a supervised four week forearm exercise training programme (3 sessions / week). Each training session involved 3 sets of unilateral dynamic handgrip exercise at 40% 1RM, at a frequency of 20 contractions per minute (duty cycle of 2 s contraction / 1 s relaxation) performed to volitional fatigue, each separated by 1 min rest. Participants exercised first with BFR before completing a work (repetition) matched protocol in the contralateral arm without blood flow restriction (CON). BFR was applied in a counterbalanced manner to the dominant or non-dominant arm. Partial BFR was induced by inflation of an 11 cm pneumatic cuff (SC10TM segmental pressure cuff, Hokanson, WA) on the upper arm to 80 mmHg. Pressure was maintained for the duration of the 3 sets, including the 1 min intervening rest periods, and amounted to 510 ± 164 s under BFR.

3.2.9 Statistics

A Shapiro-Wilk test was used to confirm normal distribution and a Mauchley test of sphericity to verify homogeneity of variance. Initially, a two way (2x3) ANOVA with repeated measures was conducted to analyse the within-subject effect of exercise condition

(BFR, CON) and time (pre-train, post-train, de-train). This was followed by one-way repeated measures ANOVA to confirm change over time in each arm separately. Bonferroni post hoc t-tests were then used to locate significance. All data are presented mean \pm SD. Significance was accepted at $P < 0.05$.

3.3 Results

All participants successfully completed the 12 training sessions with 100% compliance. HR and blood pressure during dynamic handgrip exercise was similar between conditions and throughout training. Exercise to volitional fatigue in the BFR arm (which was matched by the CON arm) progressively increased over the training period (451 ± 99 s; 477 ± 112 s; 522 ± 173 s; 586 ± 220 s, Week 1-4 respectively). All baseline (pre-training) variables were similar between the BFR and CON arms.

3.3.1 Brachial artery measures

Resting diameter, blood flow and shear rate Resting diameter increased post-training and decreased after de-training in the BFR but not the CON arm (condition x time interaction; $P = 0.019$, Figure 3.1). In the BFR arm resting diameter increased by 3.0% following training (Bonferroni t-test, $P = 0.03$) and returned to near baseline values after detraining. In the CON arm, there were no changes in resting diameter at any time point. Resting blood flow and SR did not change at any time point in either arm (Table 3.1).

Flow mediated dilation (FMD) In the BFR arm, absolute peak diameter (Figure 3.2) and peak blood flow increased by 2.4% and 18.3%, respectively, post-training (Bonferroni t-test, $P = 0.049$ and $P = 0.004$, respectively), before returning to near baseline values following detraining (Table 3.1). In the CON arm, there were no changes in absolute peak diameter or peak blood flow at any time point (Table 3.1). There were no changes in FMD [absolute (mm) and relative (%)] or shear stimulus (SR_{AUC}) in either arm at any time point (Table 3.1). Since a significant correlation between FMD% and SR_{AUC} existed (Pearson correlation, $r = .35$, $P = 0.01$), normalised FMD for shear was calculated, but no changes were observed.

Dilatory Capacity (DC) In the BFR arm, maximal diameter (Figure 3.3) increased by 3.1 % following training (Bonferroni t-test, $P = 0.02$) and returned to near baseline values after

detraining. In the CON arm, maximal diameter changed over time but post hoc analysis failed to locate significance (Table 3.1). There was a significant time effect for DC (Two-way ANOVA; $P = 0.05$), but no changes in each arm independently (Table 3.1) were observed.

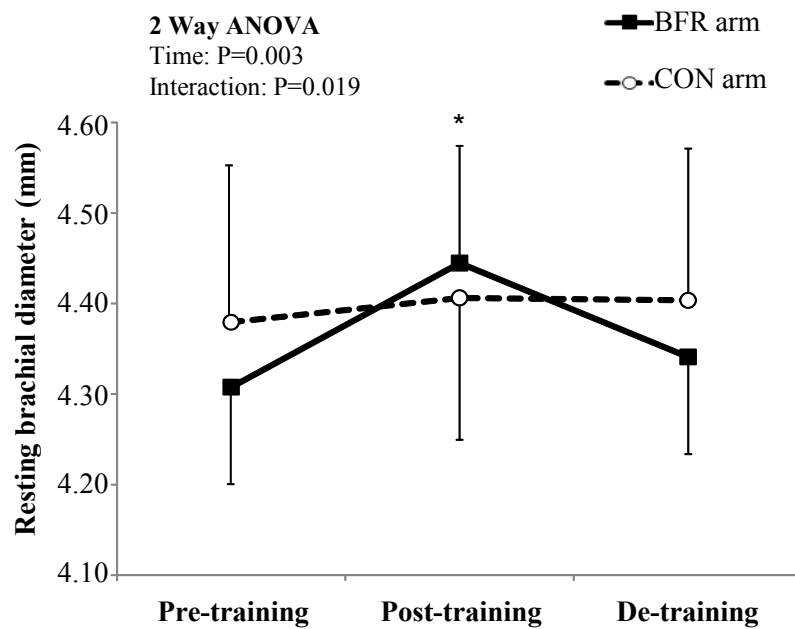


Figure 3.1 Resting brachial artery diameter measured pre-training (week 0), post-training (week 4) and de-training (week 6) in blood flow restricted (BFR) and non restricted (CON) arms ($n=9$). Values are means \pm SEM, * Significant ($P<0.05$) difference to pre-training (Bonferroni t-test).

Table 3.1 Brachial artery characteristics measured pretraining (week 0), posttraining (week 4) and detraining (week 6) in blood flow restricted (BFR) and nonrestricted (CON) arms (n=9)

	BFR arm			CON arm				
	Pretraining	Posttraining	Detraining	ANOVA	Pretraining	Posttraining	Detraining	ANOVA
<i>Resting</i>								
Diameter, mm	4.31 ± 0.28	4.44 ± 0.37*	4.34 ± 0.30	0.005	4.38 ± 0.47	4.41 ± 0.44	4.40 ± 0.45	0.11
BF, ml·min ⁻¹	160 ± 67	172 ± 76	154 ± 60	0.28	131 ± 28	133 ± 30	135 ± 23	0.94
SR, s ⁻¹	167 ± 51	69 ± 55	159 ± 60	0.80	138 ± 38	137 ± 44	143 ± 52	0.89
<i>Response to 5-min ischemia</i>								
Peak diameter	4.59 ± 0.32	4.70 ± 0.39*	4.63 ± 0.31	0.015	4.68 ± 0.44	4.69 ± 0.43	4.68 ± 0.42	0.82
Peak BF	897 ± 286	1061 ± 247†	999 ± 250	0.02	900 ± 304	910 ± 244	994 ± 239	0.46
FMD, %	6.5 ± 2.7	5.7 ± 2.0	6.7 ± 2.4	0.14	6.9 ± 1.6	6.6 ± 2.6	6.3 ± 2.5	0.48
SR _{AUC}	24,707 ± 9,881	24,509 ± 6,543	23,453 ± 5,621	0.86	21,673 ± 6,239	23,727 ± 5,178	19,782 ± 8,801	0.46
<i>Response to 5-min ischemic exercise</i>								
Max diameter	4.82 ± 0.29	4.97 ± 0.30*	4.84 ± 0.30	0.004	4.87 ± 0.39	4.93 ± 0.36	4.87 ± 0.39	0.03
DC, %	12.0 ± 3.7	12.6 ± 3.3	12.0 ± 3.3	0.62	12.1 ± 2.4	12.8 ± 3.3	11.1 ± 3.2	0.09

Values are means ± SD. Significant difference to pre-training at * P<0.05 or †P<0.005 (Bonferroni t-test). BF indicates blood flow; SR, shear rate; FMD, flow mediated dilation; SR_{AUC}, shear rate area under the curve; DC, dilatatory capacity.

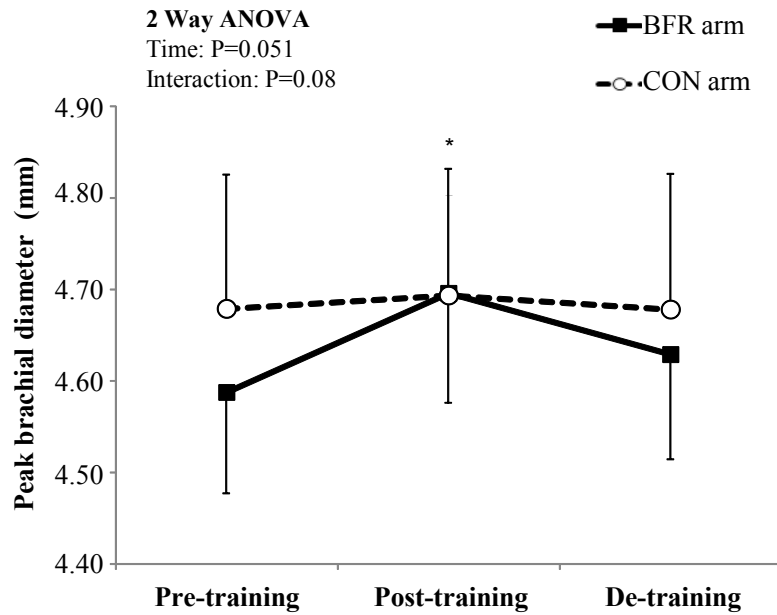


Figure 3.2 Peak brachial artery diameter measured pre-training (week 0), post-training (week 4) and de-training (week 6) in blood flow restricted (BFR) and nonrestricted (CON) arms. ($n=9$) Values are means \pm SEM, * Significant ($P<0.05$) difference to pre-training (Bonferroni t-test).

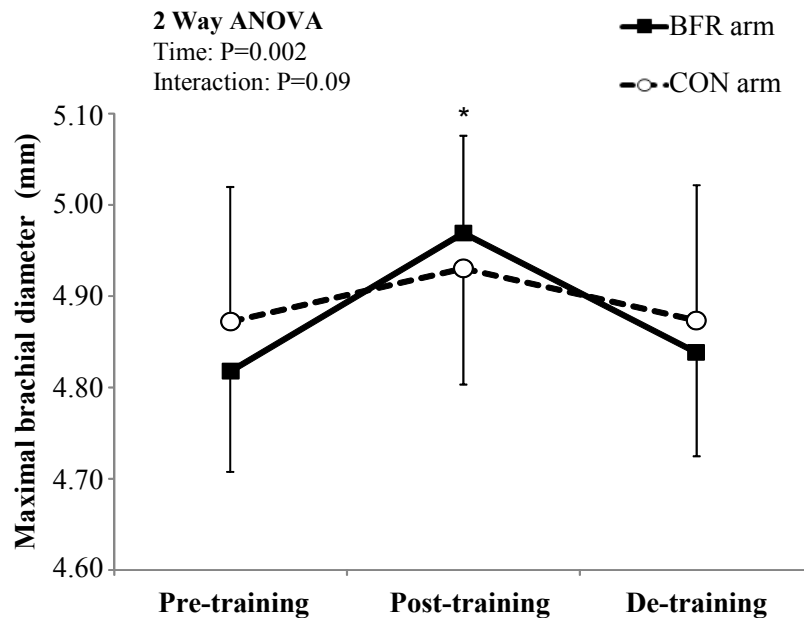


Figure 3.3 Maximal brachial artery diameter measured pre-training (week 0), post-training (week 4) and de-training (week 6) in blood flow restricted (BFR) and nonrestricted (CON) arms ($n=9$). Values are means \pm SEM, * Significant ($P<0.05$) difference to pre-training (Bonferroni t-test).

3.3.2 Peripheral vascular measures

Resting forearm blood flow decreased after de-training compared to post-training (Two-way ANOVA, $P = 0.03$) however, this was significant in the CON arm only (Bonferroni t-test, $P = 0.03$, Table 3.2). Peak post-occlusion blood flow increased post-training and decreased after de-training in the BFR but not the CON arm (condition x time interaction, $P = 0.018$). In the BFR arm, peak post occlusion blood flow increased by 28.7% following training (Bonferroni t-test, $P = 0.03$) and returned to near baseline values after de-training (Table 3.2). In the CON arm, there were no changes in peak post-occlusion blood flow at any time point (Table 3.2).

3.3.3 Forearm strength and anthropometry

1-RM increased post-training in both arms (Two-way ANOVA, $P < 0.001$, Table 3.3). There were no changes in forearm volume or circumference after training (Table 3.3).

Table 3.2 Forearm blood flow measured pre-training (week 0), posttraining (week 4) and detraining (week 6) in blood flow restricted (BFR) and non restricted (CON) arms ($n=9$)

	BFR arm				CON arm			
	Pretraining	Posttraining	Detraining	ANOVA	Pretraining	Posttraining	Detraining	ANOVA
R_{bf} , ml·min ⁻¹ ·100ml ⁻¹	1.7 ± 0.5	1.8 ± 0.5	1.4 ± 0.2	0.06	1.9 ± 0.6	1.9 ± 0.4	1.5 ± 0.4^	0.05
PO_{bf} , ml·min ⁻¹ ·100ml ⁻¹	30.3 ± 4.3	39.0 ± 5.8*	29.2 ± 8.0	0.001	29.3 ± 7.0	31.9 ± 7.5	28.2 ± 6.4	0.15

Values are means ± SD. Significant difference to pre-training * and post-training [^] at * $P<0.05$. R_{bf} , resting blood flow; PO_{bf} post-occlusion blood flow.

Table 3.3 Forearm strength and anthropometry measured pretraining (week 0), posttraining (week 4) and detraining (week 6) in blood flow restricted (BFR) and nonrestricted (CON) arms ($n=9$)

	BFR arm			CON arm				
	Pretraining	Posttraining	Detraining	ANOVA	Pretraining	Posttraining	Detraining	ANOVA
1RM, kg	42 ± 8	47 ± 9*	45 ± 8	0.001	42 ± 8	47 ± 8*	45 ± 8	0.002
Forearm volume, ml	1093 ± 104	1112 ± 131		0.32	1109 ± 89	1099 ± 122		0.70
Forearm circumference, cm	27.9 ± 1.2	28.1 ± 1.3		0.17	28.0 ± 1.2	28.1 ± 1.4		0.56

Values are means ± SD. Significant difference to pre-training at * $P<0.005$ (Bonferroni t-test). 1RM; 1 repetition maximum.

3.4 Discussion

This study has demonstrated that 4 weeks of dynamic low load handgrip training with BFR increased brachial artery diameter at rest, in response to ischemia (peak diameter) and ischemic exercise (maximal diameter), and enhanced forearm reactive hyperaemic blood flow, compared to no changes with low load training alone. Due to the similar increases in resting and stimulated diameters, FMD% and DC% remained unchanged. Taken together, these findings suggest that dynamic low load handgrip training with BFR induces adaptations within the conduit artery and peripheral resistance vessels. These adaptations were transient as all measures returned to pre-training values after two weeks de-training.

There was a 3% increase in resting diameter of the brachial artery which may reflect functional influences on vascular tone as well as an increase in artery size (Naylor *et al.*, 2005). Changes in basal vascular tone are likely to be locally mediated (Alomari & Welsch, 2007) by myogenic mechanisms and endothelial factors (such as NO and endothelin-1 bioavailability) which act on underlying vascular smooth muscle (Levick, 2011). The enhanced expression of VEGF which has been observed following BFR exercise (Takano *et al.*, 2005) may modulate NO bioavailability (Servos *et al.*, 1999; Pritchard *et al.*, 2001) therefore reducing vascular tone. Alternatively, the increase in resting diameter may be explained by artery remodelling. Indeed, the concurrent increase in maximal diameter (evoked by ischemic exercise) suggests structural enlargement in the brachial artery of the BFR arm (Naylor *et al.*, 2005) likely mediated by exposure to excessive shear, transmural and metabolic stress during exercise (Adair *et al.*, 1990; Laughlin *et al.*, 2008).

Recent studies investigating conduit artery adaptations to exercise training have used a form of blood flow restriction (Tinken *et al.*, 2010; Thijssen *et al.*, 2011b) specifically designed to manipulate exercise-induced shear rate. This was achieved by inflating a distal (forearm) cuff at a low pressure (60 mmHg). The attenuation of brachial artery shear rate in this manner during endurance-type (30 min) handgrip training actually prevented functional change (FMD) and structural enlargement (DC) (Tinken *et al.*, 2010) but not wall remodelling (Thijssen *et al.*, 2011b). In contrast, the aim of the present study was to assess brachial artery adaptation to “traditional” BFR

resistance exercise. Subsequently, we positioned the cuff proximal to the exercising muscle (on the upper arm) and inflated to a higher pressure in order to occlude venous flow before commencing a low load resistance exercise bout. Indeed, our training load was even lower (<10 min at 40% 1RM, 3 d/wk) compared to those commonly employed to mediate vascular adaptation in healthy populations (20-30 min at 40-75% MVC, 3-5 d/wk, Alomari *et al.*, 2001; Allen *et al.*, 2003; Tinken *et al.*, 2010; Thijssen *et al.*, 2011b). This may explain why artery function and structure in the uncuffed 'control' arm remained unchanged in contrast to the aforementioned studies. Enlargement of the brachial artery in the BFR arm, despite the low training load (which was matched between arms), demonstrates the capacity for the BFR stimulus to augment the training response. Our findings may be due to the shear stress profile elicited during BFR handgrip exercise (Credeur *et al.*, 2010). Oscillatory shear conditions are thought to increase adhesion molecules on the endothelial cell (Chappell *et al.*, 1998; Hwang *et al.*, 2003), which play a pivotal role in the initial stages of arterial remodelling (Heil & Schaper, 2004; Silver & Vita, 2006). Alternatively, structural remodelling may have occurred by mechanisms independent of shear stress.

Forearm exercise with proximal BFR (cuff placed on the upper arm) exposes the assessed brachial region to ischemic and/or metabolic factors (Takarada *et al.*, 2000b). An enhanced contribution of NO to conduit artery vasodilation occurs under these conditions (hypoperfusion) compared with exercise under normal inflow (Casey & Joyner, 2009; Casey *et al.*, 2010), potentially augmenting the stimulus for structural adaptation. The enhanced release of metabolites (e.g. adenosine), endothelial progenitor cells (EPCs) and VEGF with ischemic exercise (Takano *et al.*, 2005; Sandri *et al.*, 2011; Casey & Joyner, 2011) may also stimulate vascular growth, although the exact contribution to conduit arterial remodelling is at present unknown (Prior *et al.*, 2004).

Our results could directly relate to the impact of the overlying cuff. External compression resulting in an increase in extravascular pressure causes a reduction in diameter and transmural pressure of the underlying brachial artery (Bank *et al.*, 1995). The decrease in diameter may elevate wall shear stress while the increase in artery distensibility (with reduced transmural pressure; Zheng & Murray, 2011) combined with exercise induced-pressure changes, may enhance cyclic stretch. These

mechanical forces stimulate the ECs to release NO (Laughlin *et al.*, 2008) and could initiate vessel remodelling. However, we are unable to confirm the mechanical forces acting on the vessel underlying the cuff without intravascular ultrasound. Another possibility is that regional increases in blood flow immediately following BFR exercise could explain brachial artery remodelling. Enhanced hyperaemia is observed after BFR, compared with non-BFR exercise (Osada *et al.*, 2003), stimulating greater FMD of the upstream conduit vessel (Agewall *et al.*, 1999). Repeated exposure to this reperfusion may increase shear stress on the arterial walls under the cuff, stimulating structural remodelling of the conduit artery.

Increases in baseline diameter can impact on the magnitude of FMD and DC change. Indeed, the magnitude of endothelium-dependent (FMD) and independent (glyceryl trinitrate) vasodilation is inversely correlated with baseline artery size which reflects the reduction in shear rate stimulus and wall-to-lumen ratio associated with artery enlargement (Schroeder *et al.*, 2000; Thijssen *et al.*, 2008). The structural adaptation in the BFR arm may have obviated the need for ongoing functional adaptations (Tinken *et al.*, 2008). Previous studies have demonstrated that functional changes in the conduit artery occur rapidly but are short lived as the artery enlarges to mitigate the stresses placed upon it. Indeed, brachial artery FMD increases after just 1 week of handgrip training (Allen *et al.*, 2003) before peaking and decreasing after a further 2 weeks (Tinken *et al.*, 2010). It is reasonable to speculate that BFR exercise induced an increase in NO bioavailability which led to structural enlargement before normalisation of circumferential wall and shear stress returned NO-dependent function to baseline levels. As a result we did not demonstrate a change in the shear rate stimulus or FMD response post BFR training. This is in contrast with Credeur *et al.* (2010) who observed a reduced FMD after BFR training despite higher SR_{AUC} , suggesting a blunted vasodilatory response to a greater shear stimulus. The reduced FMD observed by Credeur *et al.* (2010) may, however, be a result of increased levels of oxidative stress which is implicated in endothelium dysfunction through decreased NO bioavailability (Förstermann, 2006; Higashi *et al.*, 2009). This is likely to have originated from the heavy training load (60% MVC) and prolonged blood flow restriction (20 min) which is greater than typical BFR resistance exercise protocols (<10-min at 20-40% 1RM). Short duration (<10 min) exercise protocols under partial vascular occlusion, such as that used in the present study, result in only modest

production of ROS (Takarada *et al.*, 2000b; Goldfarb *et al.*, 2008) which in fact may be involved in the vascular remodelling process, since it is now becoming accepted that repeated exposure to moderate levels of exercise induced ROS can confer beneficial adaptive signalling responses (Ushio-Fukai, 2006; Suvorava & Kojda, 2007).

Resting forearm blood flow was not affected by exercise training, which is consistent with previous reports (Green *et al.*, 1994; Alomari & Welsch, 2007; Alomari *et al.*, 2010). The 29% improvement in the peak post occlusion (reactive hyperaemic) blood flow after BFR training supports previous observations in the calf muscle group (Patterson & Ferguson, 2010), and compares favourably with other handgrip training studies (Alomari & Welsch, 2007; Alomari *et al.*, 2010). The increase in peak reactive hyperaemia represents enhanced resistance vessel vasodilatory capacity, which may be explained by improved endothelial function (Meredith *et al.*, 1996). However, adaptations are likely to extend beyond the endothelium as NO contributes mainly to the total rather than the peak reactive hyperaemic response (Tagawa *et al.*, 1994; Engelke *et al.*, 1996). The increase in resistance vessel dilatory capacity may alternatively be due to enhanced sensitivity of resistance vessels to metabolic vasodilators such as adenosine (Laughlin & McAllister, 1992; Hambrecht *et al.*, 2000) or structural remodelling (Prior *et al.*, 2004).

Both brachial artery diameters (resting and maximal) and forearm peak post occlusion blood flow returned to baseline values 2 weeks after the cessation of BFR training. Similar changes in diameter were observed in the femoral artery in response to unilateral leg endurance training and detraining (Miyachi *et al.*, 2001). While Alomari *et al.* (2010) observed the rapid return of forearm reactive hyperaemic blood flow to pre-training levels 1 week after the cessation of 4 weeks handgrip training. Inward remodelling of the conduit and resistance vessels may occur due to the removal of pulsatile stretch and shear stress on the arterial wall that would normally occur during muscle contractions (Alomari *et al.*, 2010).

The increases in strength were the same (12%) following handgrip exercise training with and without BFR. This is surprising since BFR has consistently been shown to enhance gains in strength with low load exercise training (Shinohara *et al.*, 1998; Evans *et al.*, 2010; Patterson & Ferguson, 2010). Our results are similar to

Burgomaster et al (2003) who did not observe any differences in strength between the BFR and control arm after bicep training at 50% 1RM. Similar gains in strength between the BFR and CON arm indicate a cross-transfer effect (Nemet *et al.*, 2002; Madarame *et al.*, 2008) and a reduced effect of BFR on the stimulus for increases in strength when exercising at moderate to high intensities (>40-50%1RM) (Takarada *et al.*, 2000b; Yamada *et al.*, 2004; Wernbom *et al.*, 2006; Laurentino *et al.*, 2008).

In conclusion, the present study has demonstrated that four weeks of dynamic low load handgrip training with BFR resulted in brachial artery structural modifications as indicated by increases in resting and maximal diameters. Enhanced peak reactive hyperaemia may indicate further arterial remodelling of the downstream resistance vessels. The rapid return of vascular measures following the cessation of BFR exercise demonstrates the sensitivity of the BFR stimulus. In order to confirm the mechanisms responsible for the peripheral vascular adaptations future studies require infusion protocols to directly examine smooth muscle and endothelial health in response to BFR exercise training.

Chapter 4: Time course of regional vascular adaptations to low load resistance training with blood flow restriction

4.1 Introduction

Peripheral vascular changes occur as an adaptive response to exercise training. Distinct mechanisms account for vascular adaptations at the different levels of the arterial tree (Green *et al.*, 2004b; Jasperse & Laughlin, 2006; Dhindsa *et al.*, 2008; Thijssen *et al.*, 2010). Shear stress is considered to be the principal mediator of changes to conduit and resistance vessel function and structure (Tuttle *et al.*, 2001; Tinken *et al.*, 2010; Birk *et al.*, 2012), while metabolic activity, mechanical overload and reduced oxygen tension contribute to angiogenesis in the vasculature of the active muscle bed (Egginton, 2009; Adair & Montani, 2010). Consequently the magnitude and location of vascular adaptation are dependent on the intensity, volume and/or mode of training (Green *et al.*, 2011). One exercise modality that challenges these concepts by distorting the stimuli associated with vascular adaptation is BFR resistance exercise performed at low loads. This type of exercise induces a similar metabolic stress as heavy load resistance exercise (Suga *et al.*, 2010) whilst altering hemodynamic (Takano *et al.*, 2005; Cheng *et al.*, 2005) and ischemic/redox signals (Kawada, 2005; Clanton, 2007) in different vascular territories. Despite a growing interest in this exercise modality the vascular adaptations remain incompletely understood.

Systemic reductions in blood pressure following BFR training suggest a decrease in peripheral artery resistance (Satoh, 2011), mediated through enhanced vessel functional dilation and/or structural remodelling. Indeed, local adaptations in the peripheral vasculature are evident from the increase in conduit artery maximal dilation (Hunt *et al.*, 2012), reactive hyperaemic blood flow (Patterson & Ferguson, 2010; Fahs *et al.*, 2011) and capillary filtration (Evans *et al.*, 2010) following low load resistance training with BFR. Although identified independently no study has collectively assessed changes at the conduit, resistance and capillary levels. Concurrent examinations of the conduit (diameter), resistance (peak reactive hyperemic blood flow) and capillary (capillary filtration) vessels will clarify if the

adaptations to BFR training are dependent on the position in the vascular tree (Green *et al.*, 2004b) and/or relative to cuff placement.

The process of conduit artery adaptation to exercise training, originally identified in animal models, depicts an early increase in endothelium-mediated vasodilation followed by a return to baseline due to arterial remodelling and structural normalisation of shear (Laughlin, 1995). This time-course of adaptation was recently confirmed in the human brachial and popliteal arteries in response to aerobic-type exercise with transient improvements in FMD followed by enhanced maximal DC, a verified index of conduit structure (Tinken *et al.*, 2008, 2010). Changes in conduit artery function have not been observed in response to resistance training at high loads (Rakobowchuk *et al.*, 2005) and low loads with BFR (Hunt *et al.*, 2012) despite evidence of arterial remodelling. However, these studies did not make frequent measurements of artery structure and function. Repeated measures are therefore required to determine if changes in conduit endothelial function precede structural remodelling or alternative mechanisms of adaptation are involved.

Therefore, the main purpose of the study was to determine the vascular changes, at the conduit, resistance and capillary level, in response to BFR low load plantar flexion resistance exercise training. Plantar flexion training has been used previously in the assessment of regional vascular adaptations (Evans *et al.*, 2010; Patterson & Ferguson, 2010) and adoption of this localised exercise model would allow for insightful measures of the vasculature downstream of the occlusion cuff and at different regions of the vascular tree. Assessment of function and structural capacity of the popliteal artery and maximal vasodilator capacity of the resistance vessels are performed using Doppler ultrasound (Naylor *et al.*, 2005; Thijssen *et al.*, 2011a), while measures of capillarity of the distal limb are conducted using strain gauge plethysmography (Gamble *et al.*, 1993).

4.2 Methods

4.2.1 Participants

Eleven healthy males (age; 22 ± 3 yrs, height; 178 ± 4 cm, body mass; 78 ± 9 kg, resting mean arterial pressure 89 ± 9 mmHg) volunteered to take part in the investigation. All were habitually physically active but none were specifically resistance exercise trained. The participants were fully informed of the purposes, risks and discomforts associated with the experiment before providing written, informed consent. The study conformed to current local guidelines and the Declaration of Helsinki and was approved by Loughborough University Ethics Advisory Committee.

4.2.2 Experimental protocol

Participants were initially familiarised with the testing procedures and training devices during preliminary visits, 1 week prior to experimental measures. Baseline outcome measures on both legs were initially conducted before participants completed six weeks of unilateral dynamic plantar flexion exercise. The trained (BFR) leg was randomly assigned to the dominant or non-dominant leg and the contra-lateral leg was used as a non-exercised control (CON). The popliteal artery, of both legs, was examined using Doppler ultrasound before and at 2-week intervals throughout the 6-week intervention. Artery diameter and flow velocity were measured at rest and following 5-mins of ischemia (peak diameter) and ischemic exercise (maximal diameter) to determine FMD, DC and peak reactive hyperaemic blood flow. In addition, peripheral vascular parameters (calf filtration capacity, venous pressure, isovolumetric venous pressure and leg capacitance) and plantar flexion 1RM were assessed before and after the 6-week training intervention in both legs. All vascular measures were conducted in the morning (± 1 h) after a 10 h fast and 24 h abstinence from exercise, alcohol, tobacco and vitamin supplementation. All repeated measures were performed at a similar time of day (± 1 h), with post-training assessments conducted at least 48 h after the final exercise session.

4.2.3 Popliteal artery assessments

Doppler ultrasound imaging was performed by the same sonographer using a Toshiba Powervision 6000 with a multi-frequency linear array transducer (7-11 MHz). Participants rested prone with the ultrasound probe positioned behind the knee to obtain a longitudinal B-mode image of the popliteal artery (9-11 MHz) at 3-4 cm depth. All images of the popliteal artery were acquired proximal to the branching of the tibial artery; however distance variation between participants was required to ensure the highest possible image quality. Ultrasound settings were standardised for each individual and kept constant for repeated measures. A vascular ECG gating module (Medical Imaging Applications, LLC, Coralville, IA) triggered acquisition of the ultrasound images on the R-wave pulse of an ECG signal. Sequential end-diastolic images were stored from on-line image digitization.

Flow mediated dilation (FMD). Resting popliteal diameter and blood flow were recorded for 30 cardiac cycles following a 20 min rest period. For the ischemic FMD stimulus a pneumatic cuff (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA, USA) placed distal to the popliteal fossa was inflated to >200 mmHg. Occlusion was maintained for 5 min before rapid cuff deflation. Recording of real-time duplex imaging was resumed 15 s before deflation and continued for ~6 min post-deflation, capturing the transient changes in flow and diameter over a total of 350 cardiac cycles.

Dilatory capacity (DC). Popliteal diameter and flow were re-measured and a return to baseline values was ensured before commencing the DC protocol. For the ischemic exercise DC stimulus, the pneumatic cuff was positioned above the imaged artery at the distal one-third of the thigh (~10cm proximal to popliteal fossa). The cuff was inflated > 200mmHg with occlusion maintained for 5 min. During the middle 3 min, rhythmic ischemic plantar flexion exercise was performed using a stretch band at 20 contractions/min. Real-time duplex imaging was performed as described for FMD.

Ultrasound Data Analysis. Popliteal artery diameter and flow velocity were analysed with a custom-designed, edge detection and wall tracking software (Medical Imaging Applications, Vascular Research Tools 5). Media-to-media diastolic diameter was

measured within a specified region of interest on B-mode images. The Doppler flow velocity spectrum was traced and TAMV ($\text{cm}\cdot\text{s}^{-1}$) computed. Synchronised diameter and velocity data, sampled at 20 Hz, enabled calculation of blood flow and shear rate.

Resting diastolic diameter (mm) was averaged over 30 cardiac cycles. The dilatory response was established from smoothed data (moving average over 3 cardiac cycles) over 350 cardiac cycles. This prolonged measure (6-min) exceeded time to peak dilation previously reported in the popliteal artery (181 ± 85 s, Thijssen *et al.*, 2008; 151 ± 79 Thijssen *et al.*, 2011c), safeguarding the capture of peak and maximal diameter following FMD and DC protocols respectively. FMD and DC are presented as the absolute (mm) and relative (%) change in post-stimulus diameter; (maximum post stimulus diameter – baseline diameter)/baseline diameter. Time to peak diameter (s) was calculated from the point of cuff deflation to the maximum post-deflation diameter. Blood flow ($\text{ml}\cdot\text{min}^{-1}$) was calculated as $(\text{TAMV} \times \pi r^2) \times 60$, where r is the radius of the popliteal artery. Resting blood flow was averaged over 30 cardiac cycles. Peak reactive hyperaemic blood flow in response to the ischemic plantar flexion (DC) stimulus was defined as the highest blood flow across a 10s period following cuff deflation (Naylor *et al.*, 2005). Shear rate was derived from Poiseuillies law and calculated accordingly as $(4 \times \text{TAMV}) / \text{diameter}$. The accumulated shear stimulus contributing to the FMD response was defined as SR_{AUC} and calculated for data up to the point of peak dilation for each individual. With the apropos use of ratio normalisation ($\text{FMD}\%/\text{SR}_{\text{AUC}}$) unresolved (Thijssen *et al.*, 2011a), FMD% and SR_{AUC} were presented independently. The day-to-day coefficient of variability for popliteal artery measurements were diameter (0.5%), FMD% (17.2%), DC% (9.8%) and peak RH_{BF} (5.8%), respectively.

4.2.4 Peripheral vascular assessments

Calf filtration capacity, venous pressure, isovolumetric venous pressure and leg capacitance were determined by venous occlusion strain-gauge plethysmography (Hokanson, Bellvue, WA, USA). Participants lay supine with their calf supported above the level of the heart using a 7cm and 15cm foam block under the knee and ankle. A pneumatic cuff (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air

Source, Hokanson, WA, USA) was positioned around the thigh and a mercury strain gauge placed around the widest portion of the calf and attached to a dual channel plethysmograph (EC6 Plethysmograph, Hokanson, Bellvue, WA, USA).

Arterial blood pressure was recorded at the brachial artery using an automated sphygmomanometer. After 20 min supine rest a series of eight 4-min cumulative pressure increments of ~7-9 mmHg (not exceeding diastolic pressure) were applied to the thigh cuff. Change in calf volume following cuff inflation (resulting from venous congestion) was detected through strain gauge signals sampled on line at 100Hz (Powerlab, ADInstruments, NSW, Australia) and measured using Chart version 5 software (ADInstruments, NSW, Australia). Increments beyond isovolumetric venous pressure induced a two-phase calf volume response comprising of an initial rapid phase attributed to venous filling and a subsequent slower phase attributed to fluid filtration (Gamble *et al.*, 1993) (Figure 2.5). The fluid filtration was measured from the slope of volume change during the 2-4th min of each pressure step. This was plotted against cuff pressure and the slope of the linear relationship defined the filtration capacity filtration capacity (K_f , $\text{ml} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1} \cdot 100\text{ml tissue}^{-1} \times 10^{-3}$). This linear relationship was extrapolated to determine the isovolumetric venous pressure (mmHg) at the x-axis (cuff pressure) intercept.

The residual volume change due to venous filling (capacitance vessel filling) was determined by stripping the filtration slope from the total strain gauge response to each pressure increment. This was achieved by fitting a linear regression through data points (expressing the change in calf volume) during the 2nd-4th min of each pressure step, to determine the microvascular filtration contribution to filling, before subtracting this linear portion from the total curve to obtain a plateau representing filling from the capacitance (venous) vessels (Figure 2.5). The curvilinear relationship between venous filling volume and cuff pressure was plotted to represent a function of the vascular and surrounding tissue compliance (Figure 2.5). Venous pressure (mmHg) was estimated at the x-axis (cuff pressure) intercept by extrapolating the relationship using a curvilinear fitting routine (polynomial 2nd order) (Christ *et al.*, 1997). Leg capacitance was calculated from the sum of the residual portions attributable to venous filling and expressed as normalised volume in ml volume

change/100 ml tissue. An investigator who was blinded to participants trained versus untrained limbs conducted the plethysmography analysis.

4.2.5 Calf plantar flexion strength

Plantar-flexion 1-RM was assessed unilaterally as a concentric-only contraction performed in the supine position on a leg press machine. The load was set at ~80% of the predicted 1RM and increased by 5% with each successful attempt. A successful lift was acknowledged when the participant completed the repetition through the range of motion (~40°) at the maximum weight possible. This was assessed through horizontal displacement of the weight rack by 8-15cm, standardised to the individual. The 1-RM was achieved within 5 attempts, each being separated by 2-3 min of rest.

4.2.6 Exercise training

Participants completed a six-week supervised resistance training programme (3 sessions / week). Each training session involved 3 sets of unilateral plantar flexion exercise performed to volitional fatigue at 30% 1RM with 1-min recovery intervals. The frequency of contractions was controlled by a metronome at 20 contractions per minute (1.5 s / 1.5 s concentric/eccentric duty cycle). BFR was applied by inflating a 13 cm pneumatic cuff (SC12L™ segmental pressure cuff, Hokanson, WA) on the distal thigh to 110 mmHg. This cuff pressure induced an approximate 60% reduction in popliteal resting blood flow (Hunt et al. unpublished data). Pressure was maintained for the duration of the 3 sets (80 ± 45 reps performed in total), including the 1 min intervening rest periods, and amounted to 5:48 ± 2:00 min under BFR conditions.

4.2.7 Statistics

A Shapiro-Wilk test was used to confirm normal distribution and a Mauchley test of sphericity to verify homogeneity of variance. Initially, a two way ANOVA with repeated measures was conducted to analyse the within-subject effect of exercise condition (BFR and CON) and time (popliteal artery measures [weeks 0, 2, 4, 6];

peripheral vascular and plantar flexion 1RM measures [weeks 0, 6]). This was followed by one-way repeated measures ANOVA to confirm change over time in each leg separately. Bonferroni corrected post hoc t-tests were then used to locate significance. All data are presented as mean \pm SD, unless otherwise indicated. Significance was accepted at $P < 0.05$

4.3 Results

There was a 98% adherence to the training sessions. There were no changes in systemic systolic and diastolic blood pressure through the intervention. Acquisition of high-quality ultrasound image data was achieved for the majority. However, ultrasound images for 2 participants were noisy and suffered from artefacts which precluded continuous automated border detection throughout the image sequence. In accordance with software quality control recommendations these images were excluded from the analysis. Data on the dilatory response to 5-min ischemia and ischemic exercise is therefore presented for $n=9$. Power calculations confirmed the reduced sample size ($n=9$) was sufficient to detect a 1.5% change in FMD & DC with a 5% two-tail significance level and 80% power. Pre-training (training week 0) variables were similar between the BFR and CON legs.

4.3.1 Popliteal artery measures

There was no change in resting popliteal diameter, blood flow or shear rate throughout the intervention in the BFR trained or non-trained CON leg (Table 4.1a and b).

Table 4.1a Popliteal artery characteristics measured at week 0, 2, 4 and 6 in BFR trained leg (n=9)

Variable	Week 0	Week 2	Week 4	Week 6	ANOVA
BFR leg					
<i>Resting</i>					
Diameter, mm	5.66 ± 0.46	5.69 ± 0.38	5.72 ± 0.41	5.77 ± 0.40	0.14
BF, ml·min ⁻¹	262 ± 63	256 ± 68	254 ± 55	296 ± 94	0.40
SR, s ⁻¹	124 ± 33	115 ± 27	116 ± 32	127 ± 35	0.62
Responses to 5-min ischemia					
Peak diameter, mm	5.94 ± 0.49	6.12 ± 0.44	6.09 ± 0.45	6.09 ± 0.45	0.013
FMD, %	5.0 ± 2.1	7.6 ± 2.9*	6.6 ± 2.1*	5.7 ± 1.6	0.002
SR _{AUC} , s[10 ³]	23.6 ± 8.6	25.8 ± 11.2	24.5 ± 10.1	29.2 ± 7.3	0.34
Responses to 5-min ischemic exercise					
Max diameter, mm	6.06 ± 0.44	6.20 ± 0.42	6.22 ± 0.42	6.26 ± 0.39*	0.002
DC, %	7.3 ± 3.3	8.9 ± 2.8	9.8 ± 3.2*	9.2 ± 2.4	0.004
Peak RH _{BF_{max}} , ml·min ⁻¹	1400 ± 278	1716 ± 362	1736 ± 440	1669 ± 404	0.03

Values are means ± SD. FMD, flow-mediated dilation; SR_{AUC}, shear rate area under the curve; DC, dilatatory capacity; Peak RH, peak reactive hyperemia. Significant difference from pretraining (week 0): *P < 0.05 (Bonferroni *t*-test).

Table 4.1b Popliteal artery characteristics measured at week 0, 2, 4 and 6 in nontrained CON trained leg ($n=9$)

Variable	Week 0	Week 2	Week 4	Week 6	ANOVA
CON leg					
	Resting				
Diameter, mm	5.60 ± 0.50	5.62 ± 0.48	5.61 ± 0.49	5.61 ± 0.51	0.86
BF, ml·min ⁻¹	238 ± 38	230 ± 54	219 ± 67	219 ± 39	0.55
SR, s ⁻¹	118 ± 22	113 ± 27	111 ± 19	108 ± 24	0.65
	Responses to 5-min ischemia				
Peak diameter, mm	5.92 ± 0.52	5.95 ± 0.51	5.93 ± 0.51	5.94 ± 0.54	0.79
FMD, %	5.7 ± 1.0	5.8 ± 1.5	5.9 ± 1.1	5.9 ± 0.9	0.64
SR _{AUC} , s[10 ³]	29.6 ± 12.1	25.3 ± 9.8	29.6 ± 12.2	28.4 ± 11.7	0.65
	Responses to 5-min ischemic exercise				
Max diameter, mm	6.05 ± 0.55	6.05 ± 0.52	6.05 ± 0.55	6.01 ± 0.56	0.65
DC, %	7.7 ± 1.5	8.0 ± 1.2	8.0 ± 1.3	7.7 ± 1.3	0.57
Peak RH _{BF} , ml·min ⁻¹	1511 ± 595	1504 ± 467	1651 ± 485	1550 ± 571	0.45

Values are means ± SD. BF, blood flow; SR, shear rate; FMD, flow-mediated dilation; SR_{AUC}, shear rate area under the curve; DC, dilatatory capacity; Peak RH, peak reactive hyperemia. Significant difference from pretraining (week 0): * $P < 0.05$ (Bonferroni t -test).

FMD. Absolute (mm) and relative (%) FMD changed in the BFR trained leg with no change in the non-trained CON leg (2-way ANOVA leg x time interaction: absolute FMD, $P = 0.02$; relative FMD, $P = 0.01$). Popliteal artery FMD% increased in the BFR leg at weeks 2 and 4 (Bonferroni t-test, $P=0.002$ and $P=0.014$, respectively) before returning to near baseline values at weeks 6 (Table 4.1a). The increase in relative FMD% occurred as a consequence of enhanced peak dilation ($> \pm 0.18$ mm 95% LoA) in 7 of the 9 participants, with latter 2 experiencing a reduced resting popliteal diameter. The use of ratio normalization (FMD/SR_{AUC}) was considered unsuitable due to a lack of correlation between popliteal FMD% and SR_{AUC} at baseline (Atkinson *et al.*, 2009; Thijssen *et al.*, 2011a). Total shear-rate stimulus (SR_{AUC}) is therefore reported independently in Table 4.1a and b.

DC. Popliteal maximal diameter (mm) increased in the BFR leg after 6 weeks of training (Bonferroni t-test, $P = 0.048$) with no change in the non-trained CON leg (2-way ANOVA leg x time interaction, $P = 0.004$; Figure 4.1B). Popliteal artery DC% increased in the BFR leg at week 4 (Bonferroni t-test, $P = 0.016$) but was no longer elevated at week 6. Peak reactive hyperaemic blood flow increased in the BFR leg during the training intervention period (1-way ANOVA, $P = 0.03$). This occurred at week 2 (t-test, $P = 0.047$) but with Bonferroni correction was no longer significant (Bonferroni t-test, $P = 0.14$) (Table 4.1a). There were no changes in maximal diameter, DC% or peak reactive hyperaemia in the CON leg throughout the intervention period (Table 4.1b).

4.3.2 Peripheral vascular measures

Capillary filtration (2-way ANOVA leg x time interaction, $P = 0.043$), isovolumetric venous pressure (2-way ANOVA leg x time interaction, $P = 0.005$) and calf capacitance (t-test, $P = 0.012$) all increased in the BFR trained leg with no change in the non-trained CON leg (Table 4.2). There was no change in venous pressure in either leg

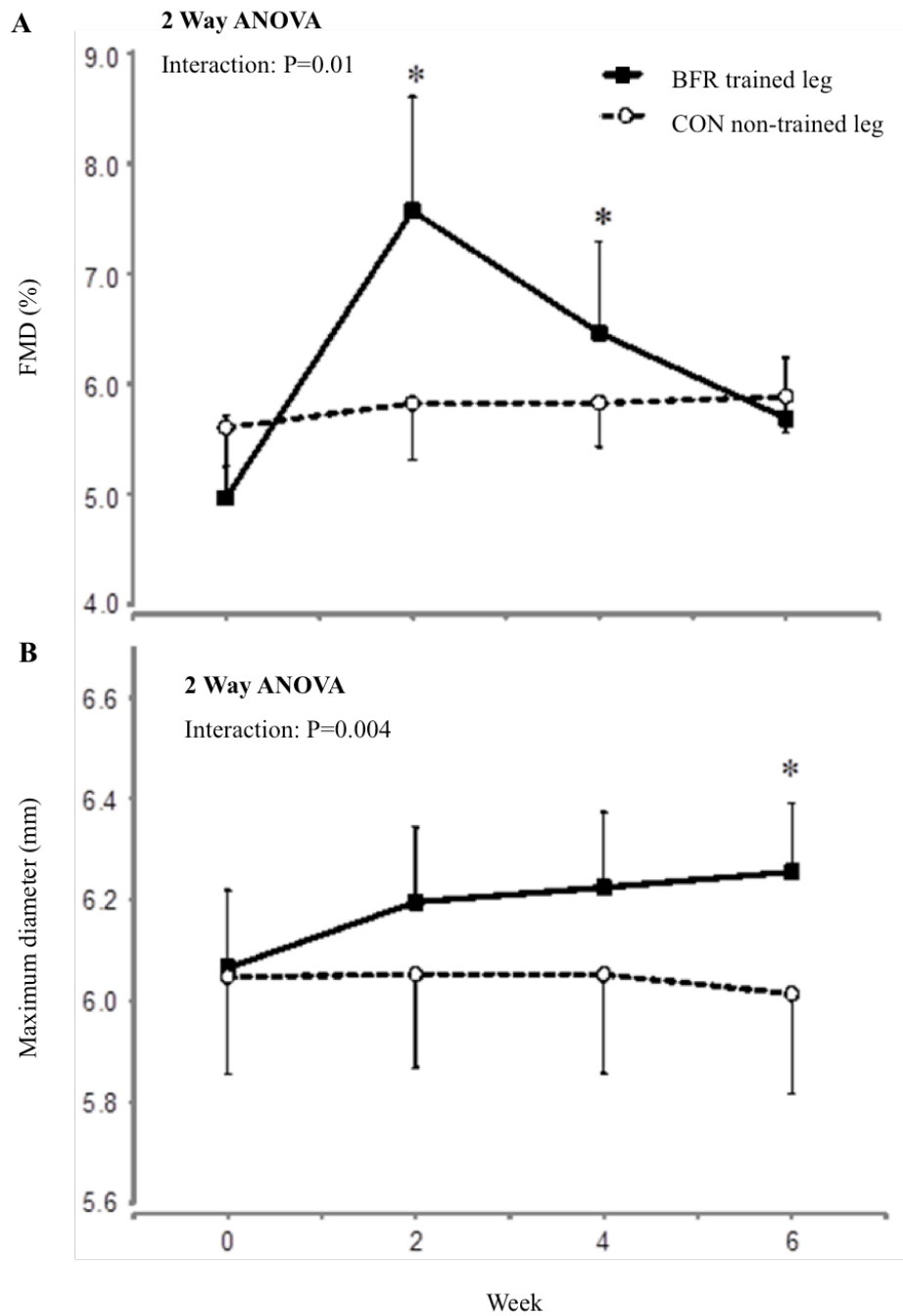


Figure 4.1 Popliteal artery (A) flow mediated dilation (FMD%) and (B) maximal diameter (mm) measured at week 0 (pre), 2, 4 and 6 (post) in BFR trained and non-trained CON legs ($n=9$). Values are means \pm SEM, Significant difference from pre-training (week 0): * $P < 0.05$ (Bonferroni t -test).

Table 4.2. Calf peripheral vascular characteristics measured before and after training in BFR trained and non-trained CON legs (*n*=11)

Variable	BFR trained		CON non-trained	
	Pre	Post	Pre	Post
K_f , ml·min ⁻¹ ·mmHg ⁻¹ ·100ml tissue ⁻¹ × 10 ⁻³	2.88 ± 0.72	3.29 ± 0.76*	2.61 ± 0.20	2.56 ± 0.25
P_{Vi} , mmHg	14.9 ± 5.6	18.3 ± 6.6*	13.4 ± 4.7	11.6 ± 4.4
P_v , mmHg	9.0 ± 3.2	9.0 ± 5.6	10.3 ± 4.7	10.5 ± 6.2
Capacitance, ml·100 ml ⁻¹	2.5 ± 0.6	3.2 ± 0.7*	2.6 ± 0.5	2.8 ± 1.2

Values are means ± SD. K_f , capillary filtration; P_v , venous pressure; P_{Vi} , isovolumetric venous pressure. * *P* < 0.05.

4.3.3 Calf plantar flexion strength

Unilateral BFR training increased 1-RM in both limbs (2 way ANOVA main effect for time, $P = 0.003$) and the effect was greater in the BFR trained (pre: $136 \pm 26\text{kg}$ vs. post: $161 \pm 27\text{kg}$) compared to the non-trained CON leg (pre: $135 \pm 21\text{kg}$ vs. post: $151 \pm 27\text{kg}$) (2 way ANOVA leg x time interaction, $P = 0.02$).

4.4 Discussion

This study has demonstrated changes at the conduit, resistance and capillary level of the vascular tree in response to unilateral low load resistance exercise with BFR with the adaptations being localized to the experimental trained leg. We observed an early increase in peak reactive hyperaemia and transient improvement in popliteal artery function before changes in artery structural capacity. This is in keeping with established models of resistance and conduit artery adaptation to non-BFR exercise (Alomari & Welsch, 2007; Tinken *et al.*, 2008). Enhanced calf filtration capacity may reflect an increased capillarity in response to BFR exercise, though the concurrent increases in isovolumetric venous pressure require further investigation.

4.4.1 Conduit artery function and structure

Unilateral BFR plantar flexion training induced only localized peripheral vascular adaptations without changes in arterial blood pressure, indicating no systemic effect and unaltered sympathetic neural control. Vascular adaptations to BFR training were therefore mediated by regional rather than systemic factors (Alomari & Welsch, 2007), as has previously been found with heavy load resistance exercise (Heffernan *et al.*, 2006). However, there appears to be no alteration in locally mediated vascular tone as basal popliteal artery parameters were unchanged in the BFR trained leg.

Popliteal artery FMD% in the BFR-trained leg increased at week 2, before returning to baseline values at week 6. This parallels the time course of transient functional adaptation in response to localized exercise (Allen *et al.*, 2003; Tinken *et al.*, 2008).

Presentation of un-normalised FMD% was advocated by the lack of correlation between FMD% and the SR_{AUC} stimulus at baseline (Atkinson *et al.*, 2009), which has been previously observed in the popliteal artery (Thijssen *et al.*, 2008). The superseding increase in popliteal maximal dilation, which is itself dependent on NO (Rudic *et al.*, 1998; Lloyd *et al.*, 2001), suggests that the transient improvements in FMD were due to enhanced intrinsic endothelial function, as opposed to stimulus magnitude.

Shear stress is thought to be the key stimulus for local exercise-induced adaptation in conduit artery function. Regular exposure to increased shear stress on endothelial cells results from increases in blood flow during exercise and is shown to alter endothelial cell phenotype, increasing expression and activity of eNOS and NO bioavailability (Laughlin *et al.*, 2008). Popliteal blood flow has been shown to be reduced with BFR (110mmHg) at rest by approximately 60%, with shear rate declining by approximately 58% (Hunt *et al.* unpublished data) and most likely remained depressed during exercise as demonstrated previously (Takano *et al.*, 2005). The shear stress stimulus during BFR exercise is therefore unlikely to have reached the threshold thought necessary to induce functional adaptation (Birk *et al.*, 2012). The impact of BFR exercise on the hemodynamic profile should also be considered. Oscillatory shear conditions activate the endothelium (Jenkins *et al.*, 2013), eliciting NO release (Green *et al.*, 2005) but with associated elevated ROS production (Laughlin *et al.*, 2008). This induces a transient decrease in FMD but is not necessarily associated with down-regulation as an adaptive response (McGowan *et al.*, 2006; Suvorava & Kojda, 2007). Indeed, the present investigation demonstrates no adverse effect on endothelial function in accordance with the low levels of oxidative stress (Goldfarb *et al.*, 2008) and inflammation (Takarada *et al.*, 2000a) associated with short duration low intensity BFR exercise.

Notwithstanding the importance of shear stress during exercise, there are other potentially relevant mechanisms that could contribute to the transient improvement in vascular function. Calf exercise with proximal BFR exposes the popliteal artery endothelial cells to ischemia. An enhanced contribution of NO to conduit-artery vasodilation occurs under these conditions (hypoperfusion) compared with exercise under normal inflow (Casey & Joyner, 2009; Casey *et al.*, 2010) augmenting the up-regulation of eNOS. This, combined with the protective effects of ischemic

preconditioning (Bailey *et al.*, 2012; He *et al.*, 2013) may contribute to an increase in NO bioavailability and endothelial function (Kimura *et al.*, 2007). Similarly the increased release of EPCs with ischemic exercise (Sandri *et al.*, 2005) may promote vascular repair (Zampetaki *et al.*, 2008). Although this represents a systemic circulatory response, mobilized EPC bind more readily to activated endothelial cells (Zampetaki *et al.*, 2008), potentially accounting for the localized improvement in FMD in the BFR trained leg only.

An alternative mechanism occurring post occlusion is reactive hyperemia, inducing an increase in antegrade shear stress and greater FMD of the upstream conduit vessel (Agewall *et al.*, 2002). Blood flow can remain elevated (2-fold above rest) for more than 1-hour post BFR exercise (Gundermann *et al.*, 2012) demonstrating the prolonged nature of this shear stimulus, which with repeated exposure may mediate endothelial adaptation. Although we have addressed the mechanisms which could account for improved popliteal artery function in spite of reductions in shear magnitude during BFR exercise, we are limited in our discussion in light of the descriptive data we have available. Future studies investigating vascular adaptations to BFR exercise should address these mechanistic issues.

The decline in popliteal FMD% was accompanied by an increase in DC% (week 4) suggesting improvement in NO-independent endothelial function and/or structural remodelling (Naylor *et al.*, 2005). Enhanced DC% may be attributed to increased production and/or sensitivity of further endothelial derived vasodilators, such as prostaglandins and EDHF (Lopez *et al.*, 2012). The return of FMD% to baseline and plateau in DC% was accompanied by an increase in maximal diameter of the popliteal artery (week 6) suggesting a structural enlargement of the conduit vessel, which obviated the need for ongoing functional adaptation (Green *et al.*, 2004a). This conforms to the theory that structural remodelling normalises circumferential wall and shear stress and returns endothelial function to baseline (Laughlin, 1995; Prior *et al.*, 2004; Tinken *et al.*, 2008).

4.4.2 Resistance vessel capacity

An increase in peak reactive hyperaemic blood flow was detected in the BFR-trained leg however post-hoc Bonferroni correction failed to confirm significance ($p=0.14$). Trends depict a 20% increase after 2 weeks of BFR training, followed by a more gradual rise and eventual plateau in the reactive hyperaemic response. This mimics the time course of forearm resistance vessel adaptation in response to rhythmic handgrip training (Alomari & Welsch, 2007; Alomari *et al.*, 2010). The enhancement in peak reactive hyperaemia precedes changes to conduit artery structural capacity suggesting initial remodelling occurs at the distal end of the vascular tree, which is in line with current theories (Birk *et al.*, 2012). Our measures made at the conduit artery by Doppler ultrasound are also in agreement with previous findings of increased calf post-occlusive blood flow measured by plethysmography following BFR plantar flexion training (Patterson & Ferguson, 2010).

The magnitude of post-occlusion blood flow in response to ischaemic exercise is governed by resistance vessel CSA (Wilkinson & Webb, 2001; Naylor *et al.*, 2005) and therefore influenced by vessel vasodilatory function (endothelium and/or smooth muscle) and structure, together with myogenic auto regulation and metabolite accumulation (Meredith *et al.*, 1996; Engelke *et al.*, 1996). It is unlikely that any BFR-training induced adaptation to adenosine and NO bioavailability influenced this reactive hyperaemic response (Lopez *et al.*, 2012), however contribution from other metabolic and endothelial vasodilators requires investigation. More likely our observations relate to structural modifications by virtue of increased density and diameter of smaller ($<40\text{ }\mu\text{m}$ in diameter) and larger resistance vessels respectively (White *et al.*, 1998). However, we cannot exclude the possibility that peak reactive hyperaemia increased as a response of enhanced venous compliance, through diminished venous congestion and reduced impedance to arterial inflow. BFR training can improve limb venous compliance (Iida *et al.*, 2011), and the increase in calf capacitance in the present investigation provides further support for this adaptation. Nevertheless, an increase in resistance vessel blood flow without changes to blood pressure or arterial compliance has been previously observed (Fahs *et al.*,

2011) suggesting probable structural remodelling with low load resistance training with BFR.

4.4.3 Capillarity

Plethysmography measures of calf K_f increased by 14% in the BFR trained leg with no observed effect in the untrained limb. In accordance with Starling's law K_f is dependent on microvascular wall surface area and permeability (Brown *et al.*, 2001). The strong correlation between K_f and capillary surface area in cross-sectional work (Brown *et al.*, 2001) and with adaptation to endurance training (Charles *et al.*, 2006) has validated its use as an indirect measure of capillarisation.

Although in agreement, our response was smaller than previously observed following BFR plantar flexion training (Evans *et al.*, 2010). This is likely attributable to the higher training load achieved in the latter study given the implementation of a discontinuous (deflating the cuff between sets) occlusion protocol. Physiological angiogenesis is a graded response (Egginton *et al.*, 2011b) and so the shorter intermittent periods of ischemia and repeated reperfusion, which characterize discontinuous BFR, may facilitate further angiogenesis through enhanced capillary shear stress. Alternatively exercise with continuous BFR (where the cuff remains inflated between sets) may activate angiogenesis via NO-independent pathways (Lloyd *et al.*, 2001) through reduced oxygen tension and a greater metabolic stress (Gustafsson *et al.*, 2007; Larkin *et al.*, 2012). Whilst the prevailing stimulus remains unconfirmed, VEGF is recognized as the principle mediator of the capillarisation response (Egginton, 2009). The immediate increase in circulating VEGF (Takano *et al.*, 2005) followed by up-regulation of VEGF, VEGFR-2 and HIF-1 α mRNA (Larkin *et al.*, 2012) suggests a coordinated angiogenic response to low load BFR resistance exercise. This may provide a sufficient stimulus to induce capillary growth with repeated exposure (training) (Gustafsson *et al.*, 2007).

The indirect nature of our measures means we cannot exclude the possibility of other influential factors. Since VEGF initiates the angiogenic cascade by increasing capillary permeability it is likely the latter contributes somewhat to our enhanced capillary filtration. In contrast to previous findings (Evans *et al.*, 2010) we observed an increase in isovolumetric venous pressure alongside enhanced K_f . This suggests

altered oncotic forces at the microvascular interface (Christ *et al.*, 2001), an effect of inflammation caused by the final BFR session or a result of cuff deflation protocols (involved in FMD and DC measures) preceding plethysmography measures (Bauer *et al.*, 2002).

4.4.4 Limitations

We acknowledge the limitations of the present study. With the inclusion of healthy habitually active male volunteers we cannot extrapolate our findings to more sedentary or clinical populations. We therefore only comment on the relevant application of this exercise mode in training environments, while further evidence is required for its potential use as a therapeutic treatment. Although the dilatory response to ischemic exercise is a valid indicator of conduit artery structure (Naylor *et al.*, 2005), endothelium independent testing via sublingual glyceryl trinitrate administration may have furthered the interpretation of results. In addition, the non-invasive nature of this investigation precluded measurements of circulating factors that may have potentially quantified stimuli (metabolic stress, ROS production) of BFR induced vascular adaptation. The duration of BFR training required to increase calf filtration capacity has been assessed previously (Evans *et al.*, 2010) but was undetermined in the present study. Repeated measures of calf filtration capacity are required to identify the time course of changes in capillarity in relation to conduit and resistance vessel adaptation. Finally we accept that within the confines of our research design and the adoption of a non-exercise control condition we were unable to certify the additional benefit of BFR to low load resistance exercise alone. However, our previous research (Hunt *et al.*, 2012) would suggest low intensity exercise is independently an insufficient stimulus for vasculature adaptation.

4.4.5 Conclusion

Changes throughout the peripheral vasculature are observed during 6 weeks of unilateral low load planter flexion training with BFR. Enhanced vasodilatory capacity of the resistance vessels appears to accompany an improvement in popliteal artery

function, which is then superseded by popliteal artery structural enlargement. Further downstream from the occlusion cuff enhanced calf filtration capacity and capacitance may potentially indicate increased capillarisation and venous compliance. The occurrence of these peripheral vascular adaptations, despite low training load, demonstrates the capacity for the BFR stimulus to augment the training response.

Chapter 5: The acute angiogenic transcriptional response to low load resistance exercise with blood flow restriction

5.1 Introduction

Capillary growth (angiogenesis) in skeletal muscle can be an adaptive response to exercise training. The process of angiogenesis is regulated by several angiogenic factors actioned through various signalling pathways (Egginton, 2011). Establishing this angiogenic response to acute exercise can inform the morphology and extent of capillary growth with repeated exposure (training) (Høier *et al.*, 2010; Hoier *et al.*, 2012). VEGF is the major angiogenic growth factor inducing capillary permeability and stimulating EC proliferation, migration and survival (Neufeld *et al.*, 1999). Activation of VEGF occurs principally through binding to its primary EC-specific receptor VEGFR-2 (Milkiewicz *et al.*, 2005). This targeted angiogenic response is stimulated by four physiological signals; shear stress, passive stretch of the tissue (contractile activity), low oxygen tension and metabolic activity (Egginton, 2009). The signals for capillary proliferation differ, depending on the exercise modality, while the angiogenic response is graded according to stimulus intensity (Egginton *et al.*, 2011a).

One exercise modality that can challenge these concepts by distorting the stimuli associated with angiogenesis is BFR resistance exercise performed at low loads. The restriction of blood flow during exercise is likely to reduce capillary shear stress (Hudlicka & Brown, 2009) while the low intensity and decreased time under tension (Loenneke *et al.*, 2012) minimises mechanical stretch. These stimuli play a predominate role in up-regulating eNOS and MMP gene expression, which are key features of longitudinal splitting and sprouting angiogenesis (Brown & Hudlicka, 2003; Prior *et al.*, 2004). Alternatively, BFR exercise may stimulate angiogenesis through signals of reduced oxygen tension and higher metabolic stress (Gustafsson *et al.*, 2007). The decrease in muscle oxygen levels during low load resistance exercise with BFR (Kawada, 2005; Tanimoto *et al.*, 2005) may stabilise HIF-1 α for targeted activation of VEGF mRNA. Exercise induced VEGF mRNA expression is graded by metabolic stress (Gustafsson *et al.*, 1999; Takano *et al.*, 2005) while vasodilators

produced during exercise (NO, adenosine, prostaglandins) promote VEGF efflux from skeletal muscle (Benoit *et al.*, 1999) and availability at EC-receptor sites for VEGFR-2 activation (Shen *et al.*, 1998). BFR has been shown to enhance the metabolic perturbations during low intensity exercise (Sundberg, 1994; Takano *et al.*, 2005; Reeves *et al.*, 2006; Suga *et al.*, 2009; Krstrup *et al.*, 2009) mimicking that of high intensity/heavy load resistance exercise. Metabolic sensor PGC-1 α may therefore be involved in the regulation of BFR-exercise induced angiogenesis (Arany *et al.*, 2008).

Previous studies have utilised BFR to investigate the regulation of exercise-induced angiogenesis but have used a pressure chamber to reduce blood flow (Gustafsson *et al.*, 1999, 2002, 2007; Norrbom *et al.*, 2004). Protocols have involved unilateral dynamic constant-load knee extension exercise (45 min, 60 rpm) at ~24% one leg peak load. Prolonged exercise of this nature has been possible because of the relatively small reduction in leg blood flow (15-20%) elicited by the pressure chamber. In contrast 'traditional' BFR resistance exercise is performed bilaterally, on a machine where knee extension activity is performed with an occlusion cuff inflated on the upper limbs reducing arterial blood flow by 40-60%. Given the difference in exercise duration and method/extent of reduced blood flow, traditional BFR exercise may present distinct findings. Recent accounts suggest low load BFR resistance exercise can enhance expression of some angiogenic genes (Larkin *et al.*, 2012) but further analysis is required to determine the physiological/molecular mediators of this response.

Therefore, the purpose of the study was to i) assess if low load resistance exercise with BFR elicits an angiogenic response and if so ii) identify the potential stimulus/stimuli responsible. We have chosen to examine the gene expression of VEGF and VEGFR-2 as this signalling pathway forms an integral component of the angiogenic response. The additional measures of HIF-1 α , PGC-1 α , eNOS and MMP-9 mRNA provide information on the potential ischemic, metabolic, shear and mechanical stress stimuli. We hypothesize that BFR will augment exercise-induced increases in skeletal muscle VEGF and VEGFR-2 mRNA. Up-regulation of HIF-1 α and PGC-1 α mRNA is expected to occur alongside this enhanced angiogenic response, reflecting reduced oxygen tension and higher metabolic stress during BFR exercise. In contrast, gene expression of eNOS and MMP-9 are likely to remain

unchanged given the low shear and mechanical stress occurring during low load exercise.

5.2 Methods

5.2.1 Participants

Six healthy males (age; 26 ± 2 yrs, height; 184 ± 6 cm, body mass; 83 ± 11 kg, resting mean arterial pressure 93 ± 10 mmHg) volunteered to take part in the investigation. All were habitually physically active but none were specifically resistance exercise trained. The participants were fully informed of the purposes, risks and discomforts associated with the experiment before providing written, informed consent. The study conformed to current local guidelines and the Declaration of Helsinki and was approved by Loughborough University Ethics Advisory Committee.

5.2.2 Experimental design

In a repeated measures cross-over design, participants completed bilateral low load knee extension exercise on two occasions; 1) under blood flow restricted (BFR) and 2) non-restricted blood flow (CON) conditions. Muscle biopsies were obtained before, 2 and 4 hours post exercise. A minimum of 3 weeks was allowed between experimental trials.

5.2.3 Preliminary trial

Participants attended a preliminary visit 3 weeks prior to the experimental trial to determine maximum knee extensor strength (1RM) and conduct exercise protocol familiarisation.

1RM determination. Bilateral maximum dynamic strength of the knee extensor muscles was assessed on a knee extension machine. The participants completed a warm up on a cycle ergometer (5 min at ~ 75 W) before moving to the knee extension machine to perform 8 reps at 50% and 3 reps at 70% of the predicted 1RM. The load was then set to approximately 80% of the predicted 1RM. Following each successful

lift the load was increased ~5% until the participant failed to lift the load through the entire range of motion. A 2-3 min recovery period was allowed between each effort and 1RM achieved within 5 attempts.

Experimental protocol familiarisation. Participants were then familiarised with the protocol of low load resistance exercise with BFR as described below. The number of repetitions performed (to fatigue) in the last set was recorded for replication in the experimental trials. This ensured a work matched design between BFR and non-BFR exercise conditions (CON), and enabled a counter-balanced order for the main experimental trial.

5.2.4 Experimental protocol

Participants attended the laboratory in the morning (7-8am) after a standardised breakfast, having monitored dietary intake for 48 h (for replication in the subsequent experimental trial) and refrained from exercise for 24 h. After resting in a supine position for 20 min a muscle biopsy was attained from the vastus lateralis at a location distal to the occlusion site (i.e. the occlusion cuff location during BFR exercise). This was followed by the exercise protocol which consisted of 4 sets of bilateral knee extension at 20% 1RM (1 x 30 reps, 2 x 15 reps, 1 x reps to fatigue, with 30 s recovery between sets), at a frequency of 20 contractions per minute (duty cycle of 1.5 s concentric and 1.5 s eccentric muscle actions) The number of repetitions performed in the last set was pre-determined from the preliminary visit.

In the BFR trial, 13 cm wide pneumatic cuffs (SC12LTM segmental pressure cuff, Hokanson, WA) were wrapped around the proximal part of each thigh and inflated to a pressure of 110mmHg (E20 Rapid Cuff Inflator and AG101 Cuff Inflator Air Source, Hokanson, WA). This occlusion stimulus induces an approximate 60% decrease in resting popliteal blood flow (Hunt et al, unpublished data). Pressure was applied 15s prior to commencing exercise and maintained for the duration of the 4 sets, including the 30 s intervening rest periods (total occlusion period of 5-8 min). In the CON trial, the same exercise was performed but under normal blood flow conditions without the application of pneumatic cuffs. After completion of the

exercise participants returned to the supine position and further muscle biopsies were obtained 2 and 4 hours post exercise.

5.2.5 Muscle biopsy

Muscle biopsies were obtained from the vastus lateralis muscle under local anesthesia (1% lidocaine) using the needle micro-biopsy technique (Acecut 11G Biopsy Needle, TSK). Samples (29.4 ± 8.3 mg) were immediately placed into tubes containing 500 μ l TRIzol reagent (Sigma-Aldrich 93289) and frozen in liquid nitrogen before storage at -80°C. Biopsies were alternated between legs and sites were separated by at least 3 cm as previously described (Gavin *et al.*, 2004). The resting biopsy sample was counterbalanced to dominant and non-dominant legs.

RNA isolation. Muscle samples snap frozen in 500 μ l TRIzol reagent were briefly thawed and gently homogenised over ice, using a handheld rotor-stator TissueRuptor probe (QIAGEN). The solution was separated into an aqueous phase and an organic phase following the addition of chloroform and centrifugation. RNA was isolated from the aqueous phase following precipitation with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol, dried and re-suspended in 50 μ l of RNA storage solution (Ambion® AM7001). Sample RNA concentration (631 ± 306 ng/ μ l) and purity (260/280: 2.0 ± 0.2 , 260/230: 1.6 ± 0.3) was confirmed using Spectrophotometry (Nanodrop).

Real-time qualitative RT-PCR. Muscle mRNA expression was determined using real-time PCR with primer sequences designed by SIGMA (Table 2.3). RNA was quantified fluorometrically using QuantiFast SYBR Green one step RT-PCR Kit (QIAGEN) and performed in duplicate (70ng of RNA in a total reaction volume of 20 μ l). Relative quantification was determined using the Mx3000P/Mx3005P QPCR cyclers (Stratagene, Agilent Technologies) with universal cycling conditions (one cycle at 50°C for 10 min [RT] and 95°C for 5min [PCR initial activation step], 40 cycles of denaturation at 95°C for 10s and annealing/extension at 60°C for 30s). Fluorescence was measured after each of the repeated cycles. On completion of PCR, all PCR products formed were melted to attain a melting curve profile, which confirmed

reaction specificity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene as it remained stable across time for both trials.

Results were expressed using the comparative cycle threshold (Ct) method. Post exercise values were reported as a fold difference relative to the expression of the pre-exercise values (Pre-exercise values arbitrarily set to 1). Relative fold changes were determined using the $2^{-\Delta\Delta Ct}$ method using the equation: Fold = $2^{(Ct \text{ target gene} - Ct \text{ housekeeping gene}) \text{ time x} - (Ct \text{ mean target gene} - Ct \text{ mean housekeeping gene}) \text{ baseline}}$.

5.2.6 Statistics

A Shapiro-Wilk test was used to confirm normal distribution and a Mauchley test of sphericity to verify homogeneity of variance. A two-way ANOVA with repeated measures was used to evaluate the VEGF mRNA response over time (pre, 2h & 4h post exercise) in the two exercise conditions (BFR, CON). Friedman's ANOVA was used to evaluate the remaining mRNA data (VEGFR-2, PGC-1 α , HIF-1 α , eNOS and MMP-9) to confirm change over time in each experimental trial (BFR, CON). Following a significant omnibus test, targeted pairwise comparisons were used to assess differences within trials with respect to pre-exercise and between trials at the same time point. Pairwise comparisons were conducted using Paired t-tests and Wilcoxon signed rank tests for parametric and non-parametric data, respectively. Differences were considered significant at $P < 0.05$. All data are presented as mean \pm SD, unless otherwise stated.

5.3 Results

Bilateral knee extension 1RM was 141 ± 21 kg. The exercise protocol was performed at 20% of 1RM, equating to 28 ± 4 kg. Following the first three sets of exercise being performed for 30, 15 and 15 reps, respectively, the number of repetitions completed in the final set to fatigue was 37 ± 28 , and varied greatly between individuals (range 4 - 81 reps). The total number of repetitions performed in the exercise bouts (BFR & CON) was therefore 97 ± 28 and amounted to 397 ± 85 s under occlusion.

Skeletal muscle mRNA. VEGF mRNA expression increased 2 and 4 h after BFR exercise ($P=0.016$ and $P=0.035$) with no change observed in CON (condition x time interaction, $P = 0.008$). The increase in VEGF mRNA was greater in the BFR compared to CON at 2 h ($P=0.02$) and 4 h ($P=0.012$) post-exercise (Figure 5.1A).

VEGFR-2 mRNA expression increased 2 and 4 h after BFR exercise ($P=0.028$ and $P=0.046$) with no change observed in CON ($P=0.85$). However the change in VEGFR-2 mRNA did not differ between trials (2 h, $P=0.25$; 4 h, $P=0.08$) (Figure 5.1B).

PGC-1 α mRNA expression increased 2 and 4 h after BFR exercise ($P=0.028$ and $P=0.028$) with no change observed in CON ($P=0.44$). The increase in PGC-1 α mRNA was greater in BFR compared to CON at 4 h ($P=0.046$) but not 2 h ($P= 0.07$) (Figure 5.1C).

HIF-1 α mRNA expression was increased 2 h after BFR exercise ($P=0.027$) but no difference was detected between trials. Large between-subject variability in the HIF-1 α mRNA response at 4 h after BFR exercise precluded significance ($P=0.145$) (Figure 5.1D).

eNOS mRNA expression peaked 2 h after BFR exercise ($P=0.028$) but a difference between conditions was only evident at 4 h post exercise ($P=0.046$) (Figure 5.1E).

MMP-9 mRNA expression did not change in response to exercise (Figure 5.1F).

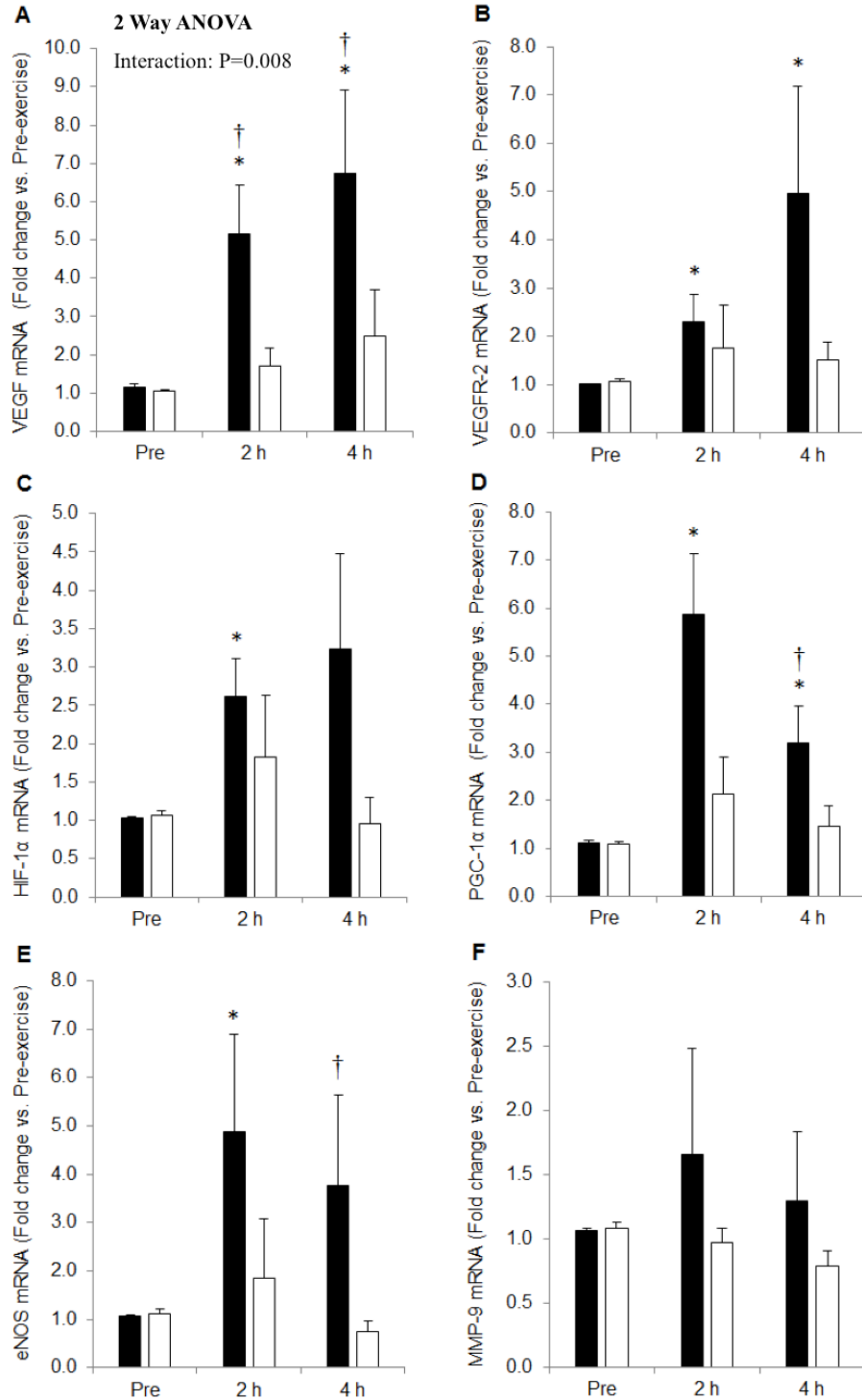


Figure 5.1 Skeletal muscle mRNA expression of vascular endothelial growth factor (VEGF, A), VEGF receptor-2 (VEGFR-2, B), peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α , C), hypoxia inducible factor 1 α (HIF-1 α , D) endothelial nitric oxide synthase (eNOS, E) and matrix metalloproteinase 9 (MMP-9, F). Changes in mRNA content are expressed as a fold change from baseline relative to GAPDH at 2 h and 4 h post blood flow restricted (BFR ■) and non-restricted (CON □) exercise. Values are means \pm SEM ($n=6$) * Significantly different to pre-exercise, † Significantly different to CON trial ($P<0.05$).

5.4 Discussion

The present study has demonstrated that BFR potentiates the expression of VEGF, PGC-1 α and eNOS mRNA in response to low load resistance exercise. Furthermore, VEGFR-2 and HIF-1 α mRNA are up-regulated following low load resistance exercise with BFR. This suggests a targeted angiogenic response to BFR exercise potentially mediated through enhanced metabolic, ischemic and shear stress stimuli. In contrast MMP-9 mRNA expression was unchanged following BFR and CON exercise which may reflect the limited development of muscular tension/stretch during low load and low volume resistance exercise protocols.

VEGF is the principle growth factor regulating angiogenesis and is therefore an integral index of angiogenic potential. The present study observed a 5.2- and 6.7-fold increase in VEGF mRNA at 2 and 4 h post BFR exercise. The time course of the increase in VEGF mRNA is similar to that reported following acute heavy load resistance exercise (Trenerry *et al.*, 2007; Gavin *et al.*, 2007), while the magnitude compares favourably with previous investigations despite lower contraction intensity (Gavin *et al.*, 2007) and shorter exercise duration (Rullman *et al.*, 2007). Although the exercise-induced increase in VEGF transcription is not a novel observation the duration of exercise in previous investigations (Gustafsson *et al.*, 1999, 2007; Ameln *et al.*, 2005) far exceeds that of our BFR protocol (e.g. 45 vs 5 min) demonstrating the efficiency of this low load resistance exercise modality. Our findings are likely attributable to the greater reduction of leg blood flow elicited by direct occlusion cuff application (60% reduced popliteal artery flow) compared to pressure chamber induced BFR (15-20% reduced femoral artery flow; Sundberg & Kaijser 1992). Concurrent changes in VEGF translation appear absent after acute BFR exercise (Gustafsson *et al.*, 2007; Larkin *et al.*, 2012) despite increased basal VEGF protein levels and indices of enhance capillarity after BFR training (Gustafsson *et al.*, 2007). This investigation may have captured a translational response in the delayed biopsy sample at 4 h post BFR exercise, but protein analysis is yet to be performed. VEGF protein is increased at this time point following non BFR exercise (Ryan *et al.*, 2006; Rullman *et al.*, 2007; Gavin *et al.*, 2007) and requires confirmation with BFR exercise.

VEGF alone is an inadequate index of angiogenic potential (Milkiewicz *et al.*, 2006). It is produced by endothelial, perivascular and skeletal muscle cells but is only biologically

activated by binding with tyrosine kinase receptors; VEGFR-1 and VEGFR-2, which are expressed predominately on ECs (Egginton, 2009). VEGFR-2 is considered the major mediator of VEGF induced angiogenesis (Milkiewicz *et al.*, 2005) and was subsequently measured in the present investigation. The 5-fold up-regulation of VEGFR-2 mRNA following low load BFR resistance exercise exceeds the responses to heavy load resistance exercise (Gavin *et al.*, 2007). VEGF can directly regulate VEGFR-2 mRNA expression in ECs (Shen *et al.*, 1998). Thus, the enhanced secretion of VEGF by muscle fibres during BFR exercise (Takano *et al.*, 2005) may explain increased VEGFR-2 expression. Alternatively the up-regulation of VEGFR-2 mRNA may result from the increased number of EC given the enhanced proliferation of EC (Gustafsson *et al.*, 2007) and EPC (Sandri *et al.*, 2011) after BFR exercise. Yet despite trends ($p=0.08$, 4 h post exercise), this VEGFR-2 transcriptional response did not differ between exercise conditions, in contrast to previous investigations (Gustafsson *et al.*, 2005; Larkin *et al.*, 2012).

Whilst VEGF and VEGFR-2 signalling is common to both modes of capillary growth, distinct angiogenic phenotypes occur as a result of differences in stimuli exposure according to the mode and intensity of exercise (Egginton, 2011). In the present study we examined the mRNA content of HIF-1 α , PGC-1 α , eNOS and MMP-9 to identify potential ischemic, metabolic, shear and/or mechanical stress stimuli.

Hypoxia-induced VEGF expression is regulated by transcription factor HIF-1 α . Measurements by near infrared spectroscopy have confirmed the magnified and prolonged decrease in muscle oxygen levels during BFR exercise (Kawada, 2005; Tanimoto *et al.*, 2005; Larkin *et al.*, 2012). Reduced oxygen tension inhibits prolyl hydroxylase, resulting in accumulation of the HIF-1 α protein and translocation into the nucleus for targeted upregulation of VEGF mRNA (Ameln *et al.*, 2005). Although BFR exercise increased HIF-1 α mRNA at 2 h (2.6 fold) the response was similar to CON. Likewise the trend at 4 h, largely driven by the robust response (>6 -fold) of two individuals to BFR exercise, was not different to CON ($p=0.17$). Collectively, these findings suggest regulation by this transcription factor does not account for the augmented VEGF expression. Whilst this contrasts with recent findings of Larkin *et al.* (2012), the majority find no difference in the activation of HIF-1 α between exercise bouts of unrestricted and restricted blood flow (Gustafsson *et al.*, 1999; Ameln *et al.*, 2005; Drummond *et al.*, 2008; Fry *et al.*, 2010), despite the latter inducing greater VEGF mRNA expression (Ameln *et al.*, 2005). Low intensity resistance exercise does not alter muscle oxygenation (Larkin *et al.* 2012) so it is

unlikely that CON exercise alone could have reduced intracellular PO₂ low enough to fully stabilize HIF-1 α . Modulation of HIF-1 α stabilization may have occurred through post-translational mechanisms and requires further analysis (Semenza, 2007b). Nonetheless, our results at present suggest that enhanced VEGF expression following BFR exercise is mediated by HIF-1 α independent mechanisms.

Transcription co-activator PGC-1 α may regulate angiogenesis in a HIF-1 α independent manner by downstream co-activation of the orphan nuclear receptor estrogen-related receptor- α (ERR- α) (Arany *et al.*, 2008). PGC-1 α mediates exercise-induced skeletal muscle VEGF expression (Leick *et al.*, 2009) which could explain the absent VEGF response to CON exercise which failed to increase PGC-1 α mRNA. As a potent metabolic sensor, PGC-1 α regulates VEGF in response to nutrient and oxygen deprivation, with expression dependent on exercise intensity (Egan *et al.*, 2010; Nordsborg *et al.*, 2010) and/or muscle fibre activation patterns (Godin *et al.*, 2010). The near 6-fold increase in PGC-1 α mRNA expression following BFR exercise may reflect the enhanced muscle fibre recruitment (Krustrup *et al.*, 2009) and exposure to heightened intramuscular metabolic stress, equivalent to that of high-intensity resistance exercise (i.e. PCr depletion, Pi splitting, pH) (Suga *et al.*, 2010). This response appears modest in comparison to prolonged (45 min – 3 h) endurance pressure chamber induced BFR exercise (7-12-fold, (Norrbon *et al.*, 2004; Watt *et al.*, 2004; Mathai *et al.*, 2008). However, it exceeds that observed after high intensity interval training (Gibala *et al.*, 2009) and heavy load resistance exercise with superimposed whole body vibration and vascular occlusion (Item *et al.*, 2012), despite similar total exercise time and lower intensity. These findings suggest that low load resistance exercise with BFR may be a modality that is metabolically effective and time efficient in up-regulating PGC-1 α . This augmented PGC-1 α response to acute exercise may encourage significant capillary growth with training (Arany *et al.*, 2008).

Shear stress is a major contributor to exercise-induced VEGF expression and is thus an important mechanical signal prompting angiogenesis within the muscle (Egginton, 2011). Dilation of the arterioles during exercise enhances capillary flow velocity and shear stress, increasing eNOS expression and NO release. A depressed response was expected during BFR exercise given the impact of reduced conduit artery blood flow on downstream capillary perfusion and shear stress (Hudlicka & Brown, 2009). Yet we observed a substantial elevation of eNOS mRNA after BFR exercise but not CON. This may be a consequence of the shear stimulus elicited by reactive hyperemia following cuff deflation with BFR exercise

(Gundermann *et al.*, 2012). However, enhanced eNOS expression is also likely due to increased circumferential strain (stretch) as a result of raised capillary pressure with proximal cuff inflation (venous occlusion) (Price & Skalak, 1994), and due to mechanical compression of the vessels with skeletal muscle contraction (Chen *et al.*, 2002). Our 4.9-fold increase in eNOS is greater than observations following acute passive knee extension exercise (Hellsten *et al.* 2008) which with training proved to be a sufficient stimulus to elevate basal eNOS mRNA and initiate capillarisation (Høier *et al.*, 2010). In contrast there was no change in eNOS mRNA in the control trial, which may reflect the modest hyperemia associated with short duration low intensity resistance exercise. Presumably this exercise dose elicited a mechanical stimulus (shear stress, circumferential strain and/or compression) that was below the threshold required to stimulate EC eNOS transcription.

The reciprocal relationship between NO and VEGF is reflected by the concurrent up-regulation of VEGF and VEGFR-2 with enhanced eNOS expression following BFR exercise. VEGF activation of VEGFR-2 can induce NO formation through calcium mobilisation and phosphorylation of eNOS (He *et al.*, 1999). However, our data supports the idea that eNOS derived NO promotes VEGF production, since peak gene expression of eNOS occurs prior to VEGF and VEGFR-2. Although it cannot be confirmed if enhanced eNOS mRNA is due to shear stress or alternative stimuli, it does suggest activation of NO pathways that initiate EC proliferation and facilitate angiogenesis by longitudinal splitting (Williams *et al.*, 2006a). However, there are limitations of conducting only gene analysis as post-translational modifications of the eNOS protein underline the dynamic regulation of enzymatic activity and in turn angiogenesis (Sessa, 2004; Michel & Vanhoutte, 2010).

While activation of eNOS is thought to be essential for angiogenesis by longitudinal splitting, proteolysis of the capillary basement membrane by MMP-9 facilitates EC migration, and is believed to be an essential component for the progression of sprouting angiogenesis (Egginton, 2011). In contrast to previous findings (Rullman *et al.*, 2007; Hoier *et al.*, 2012) there was no change in MMP-9 mRNA expression following acute exercise. This is probably due to the protocols short duration rather than low intensity, since passive (unloaded) leg exercise is sufficient to up-regulate MMP-9 gene expression (Høier *et al.*, 2010). Although Høier *et al.* (2010) utilised a similar knee extension/flexion movement pattern, the longer duration of passive exercise could have provided greater mechanical stimulation and enhanced stretch (through the shortening and lengthening of sarcomeres) of the muscle tissue.

The expression of MMP-9 mRNA did not differ between conditions which may be due to the workload match protocol eliciting a similar mechanical response in BFR and CON trials. Transcriptional activation of MMP-9 is induced by muscle damage and inflammatory cytokine response. Low load resistance exercise with BFR does not appear to elicit an inflammatory response (Takarada *et al.*, 2000a; Clark *et al.*, 2011) consistent with our lack of alteration in MMP-9 gene expression. Furthermore, our finding that BFR exercise augments PGC-1 α mRNA, but not MMP-9 is in agreement with Rullman *et al* (2009) who demonstrated the negligible role of metabolic stress in exercise induced MMP-9 expression. Although short duration low load resistance exercise does not increase MMP-9 mRNA, changes in MMP-9 activity due to post-transcriptional mechanisms cannot be excluded without zymographic analysis. Further research is required to confirm MMP-9 activity following BFR exercise since its absence can eliminate angiogenesis induced by muscle stimulation (Haas *et al.*, 2000). Indeed, coordination between growth factor and protease expression is required to avoid the loss of integrity of the microcirculation (Egginton, 2009).

Given the acute nature of this experiment, there are limitations in predicting chronic adaptations to BFR training. However, previous studies have demonstrated that the acute angiogenic response does inform the magnitude of capillary growth with training. Increases in acute exercise-induced VEGF, VEGFR-2 eNOS and MMP-9 expression are associated with a significant capillarisation response after 4 weeks of training (Hellsten *et al.*, 2008; Høier *et al.*, 2010; Hoier *et al.*, 2012). The failure to up-regulate MMP-9 in response to acute BFR exercise may obviate the progression of sprouting angiogenesis with training. However, capillary growth by longitudinal splitting is a process requiring reduced matrix remodelling and occurs independent of MMP involvement. Indeed, the enhanced expression of eNOS mRNA following acute BFR exercise demonstrates potential for shear stress dependent angiogenesis. A more detailed insight into the angiogenic cascade with measurement of further pro- and anti-angiogenic factors is required to determine the modulation and morphology of capillary growth with BFR exercise training.

Chapter 6: The effect of participant characteristics on the relationship between cuff pressure and level of blood flow restriction on the upper and lower limbs

6.1 Introduction

Blood flow restricted (BFR) exercise is a training modality generating substantial research interest. Typically, resistance exercise is performed at low loads (20-40% 1RM) to volitional fatigue with a compressive cuff inflated around the exercising limb restricting blood flow. Studies have demonstrated that this training stimulus can illicit similar hypertrophic and strength gains as traditional heavy load resistance exercise (Takarada *et al.*, 2000b; Karabulut *et al.*, 2010), improve skeletal muscle endurance (Takarada *et al.*, 2002; Loeppky *et al.*, 2005; Sumide *et al.*, 2009; Kacin & Strazar, 2011) and prompt vascular remodelling (Evans *et al.*, 2010; Hunt *et al.*, 2013). Whilst the potential benefits are clear, many studies observe a wide range of individual differences in the acute and chronic response to BFR training. The additional variables encountered with BFR exercise may be responsible for the obfuscated training outcomes.

Perhaps the most obvious consideration is the variance in the level of blood flow restriction between individuals. This not only affects the tolerance to BFR exercise and subsequent acute physiological responses, but ultimately training adaptations. It is common in BFR exercise protocols for the cuff to be inflated to the same absolute occlusion pressure (mmHg) for all individuals, but this stimulus may not reduce blood flow to the same degree in each individual. This will influence oxygen and nutrient delivery as well as accumulation and clearance rate of local metabolic by-products during exercise (Takarada *et al.*, 2000b; Yasuda *et al.*, 2008; Karabulut *et al.*, 2011). Variation in the level of BFR could therefore explain the discrepancy in exercise tolerance, which is evident in the literature from the range in total exercise duration (number of repetitions to fatigue) (Cook *et al.*, 2007) and perceived rate of exertion (Yasuda *et al.*, 2008; Karabulut *et al.*, 2011). Standardising the BFR stimulus between individuals may reduce the variability observed in response to BFR training. It would therefore be of interest to quantify the level of relative blood flow restriction over a range of absolute occlusion pressures in an attempt to determine the variation between individuals and methods for correction.

The level of BFR required to obtain the desired physiological adaptations to training is undefined. An 'appropriate' level of BFR increases muscle activation at the same external load without inducing premature fatigue (contractile/metabolic impairment) (Yasuda *et al.*, 2008). Complete arterial occlusion causes greater ratings of perceived exertion (Sumide *et al.*, 2009; Yasuda *et al.*, 2009) and limits the tolerable duration of exercise (Cook *et al.*, 2007; Yasuda *et al.*, 2009), reducing the effectiveness of BFR resistance training (Sumide *et al.*, 2009; Kacin & Strazar, 2011). Indeed, achieving a high exercise volume appears crucial, as it is the potential mediating factor for skeletal muscle hypertrophy (Burd *et al.*, 2010) and vascular remodelling (Prior *et al.*, 2003). Therefore, efficacy of BFR resistance exercise occurs with the suppression of venous outflow (causing pooling of the blood) and the partial but not complete reduction in arterial inflow. BFR training studies that have proven effective adopt an occlusion protocol eliciting an approximate 60% reduction in resting blood flow (Takano *et al.*, 2005).

The level of BFR is influenced by the tourniquet cuff design and application method. The width of the cuff is an important variable to consider. Wide cuffs transmit a greater percentage of the applied tourniquet pressure to deeper tissues than narrow cuffs and are therefore more effective in restricting arterial blood flow at lower inflation pressures (Crenshaw *et al.*, 1988). Indeed, Loenneke *et al.* (2011) found the cuff pressure required for complete arterial occlusion was significantly lower in wide (144mmHg; 13.5cm wide, Hokanson) compared to narrow (235mmHg; 5cm narrow, Kaatsu Master) cuffs prevalently used in BFR research. Wide cuffs are therefore recommended as they reduce the necessary pressure application for partial occlusion (Wernbom *et al.*, 2006). However attenuation of muscle hypertrophy (CSA) can occur at the application site suggesting a limitation of utilising a wider restrictive cuff (Kacin & Strazar, 2011).

Rarely do investigations control for individual factors that influence the relative level of BFR. For the few studies that have attempted to do so, the most common method is to adjust the cuff pressure according to individual's systolic blood pressure (~130% SBP) (Takano *et al.*, 2005; Karabulut *et al.*, 2006; Clark *et al.*, 2011; Cook *et al.*, 2010). Whilst this seems to work successfully in training studies using narrow cuffs no equivalent calculation for use with wider cuffs has been approved. Partial occlusion (~60%) is likely to occur, using wider cuffs, at much lower pressures than 130% SBP. In fact, findings by Loenneke *et al.* (2011) suggest wide cuff inflation at 130% SBP (130% of ~120 = 156 mmHg) would exceed the necessary

pressure for complete arterial occlusion (144 mmHg) in healthy individuals. Even so there is debate that SBP should even be considered since studies report a moderate ($r = 0.56$, Younger *et al.* 2004) if not absent ($r = 0.05$, Crenshaw *et al.*, 1988; Loenneke *et al.*, 2011) relationship between it and limb occlusion pressure. This suggests that basing cuff pressure on SBP alone may not lead to optimal BFR and further variables should be investigated.

The relative degree of BFR may be subject to the amount of the tissue surrounding the blood vessel as this influences the pressure exerted on the vasculature. In fact limb circumference explained most of the variance in the cuff pressure required to occlude arterial flow (Loenneke *et al.*, 2011) and may influence the fatigue response and degree of muscle hypoxia during BFR exercise (Yasuda *et al.*, 2008). On this basis a different cuff pressure would be required to elicit optimal BFR on upper and lower limbs. Applying just 50mmHg on the upper arm increases electromyographic activity during elbow flexion exercise (Takarada *et al.*, 2000b) suggesting blood flow may be restricted at lower pressures than typically observed in lower limb exercise models. Yet to our knowledge the difference in blood flow during upper and lower limb occlusion using wide cuffs has not been identified. Restrictive pressures based on limb size are advised (Fahs *et al.*, 2012b), but without reporting absolute values this study offers no means of practical application.

Less is understood on the impact of tissue type (muscle or fat) on the level of BFR. It is likely the degree of intramuscular pressure depends on the architecture features of the muscle. Hypertrophied muscles have a greater thickness and volume, and subsequently blood flow occlusion may occur at relatively lower pressures (Wernbom *et al.*, 2006). Despite this, fat as opposed to muscle CSA explained most of the variance in the cuff pressure required to occlude arterial flow (Loenneke *et al.*, 2011). It is not known how limb composition influences BFR over the range of venous occlusion pressures typically employed during training.

Although previous studies suggest adjusting the restrictive cuff pressure based on the individual's blood pressure, limb size and composition, the lack of reported values offer no means of application. Furthermore, the assumption of a linear relationship between cuff pressure and the level of BFR cannot be made. Indeed, a recent study by Laurentino *et al.* (2012) reported that 50% BFR (relative to resting blood flow) was achieved at 80% of the pressure required to occlude arterial flow, suggesting a non-linear relationship between these factors.

Therefore, the aim of this study is to establish whether subject characteristics (i.e. blood pressure, limb size and composition) should be accounted for when prescribing the cuff pressure required for BFR. The latter will be estimated by determining the relationship between cuff pressure and the level of blood flow restriction for each individual. This will be achieved by measuring the changes in conduit artery diameter and blood velocity in response to proximal cuff inflation over a range of external pressures.

6.2 Methods

6.2.1 Participants

Fifty participants (25 male, 25 female; Age; 21 ± 3 yrs, height; 176 ± 10 cm, body mass; 69.7 ± 12.1 kg) volunteered to take part in the investigation. All were fully informed of the purposes, risks and discomforts associated with the experiment before providing written consent. The study conformed to guidelines outlined in the Declaration of Helsinki and was approved by Loughborough University Ethics Advisory Committee.

6.2.2 Experimental protocol

All tests were conducted 4 h post-prandial in a quiet, temperature-controlled room ($24 \pm 1^{\circ}\text{C}$). Participants abstained from exercise, alcohol and tobacco for 24 h prior to the experimental test. Upon arrival into the laboratory, participant's height and body mass were measured using a stadiometer and manual scales. Anthropometric assessments of the right thigh and arm were made, followed by B-mode ultrasound measures of anterior and posterior subcutaneous adipose tissue thickness. Participants then rested supine for 20 min prior to arterial blood pressure measurement and vascular assessments. Doppler ultrasound was used to image the popliteal and brachial artery in response to incremental blood flow restriction induced by proximal pneumatic cuff inflation.

6.2.3 Anthropometry, adipose thickness and estimated muscle-bone CSA

Thigh circumference was measured horizontal to the long axis of the femur at the mid-point between the inguinal fold and the anterior aspect of the patella. The horizontal

(circumferential) line was marked and extended to intercept with the vertical mid-line of the anterior and posterior surface of the thigh. Subcutaneous adipose tissue thickness overlying the quadriceps and hamstrings were measured at these sites. Arm circumference was measured horizontal to the long axis of the humerus at the mid-acroniale-radiale distance. The horizontal (circumferential) line was marked and extended to intercept with the vertical mid-line of the anterior and posterior surface of the arm. Subcutaneous adipose tissue thickness overlying the biceps and triceps were measured at these sites.

Orientated in the sagittal-plane, the centre of the ultrasound transducer was aligned perpendicular to the horizontal (circumferential) line, on the anterior and posterior surface of the limb. Subcutaneous adipose tissue was imaged at depth of 2-5cm, depending on the site and individual. Minimal force was applied through the transducer to avoid compression of the adipose layer. Two ultrasound B-mode images (Toshiba Powervision 6000 with a multi-frequency linear array transducer (7-11 MHz) were systematically acquired at each site (quadriceps, hamstrings, biceps and triceps) and later analysed using Image J software. On each image, subcutaneous fat thickness was measured between skin-fat and fat-muscle interfaces at four sites and the average of eight values recorded (Figure 2.7). Limb adipose thickness (AT) was determined by averaging values attained at anterior and posterior sites [thigh AT = (quadriceps AT + hamstring AT) / 2; Arm AT = (brachial AT + triceps AT) / 2]. Muscle-bone CSA was estimated using the formula $\pi((r - AT)^2)$ where r is the radius of the limb calculated as circumference / 2π and AT is ultrasound measured adipose thickness (Bemben *et al.*, 2005).

6.2.4 Blood pressure

Systolic (SBP) and diastolic (DBP) blood pressures were measured from the brachial artery, following 5-min supine rest, using an automated blood pressure cuff (Omron M5-1 Digital BP monitor, Omron Healthcare, The Netherlands). Blood pressure was taken in triplicate and the closest two values averaged for analysis. Mean arterial pressure (MAP) was calculated as $DBP + (SBP - DBP)/3$ in accordance with Levick (2011).

6.2.5 Ultrasound measures of blood flow restriction

Popliteal artery. Participants lay in a prone position for 20 min before the test protocol commenced. A 13 cm wide pneumatic cuff (SC12LTM segmental pressure cuff, Hokanson, WA) was positioned on the right thigh overlying marks used for the prior anthropometric assessment. Popliteal artery diameter and velocity were measured using duplex ultrasound behind the popliteal fossa at depths of 3-4 cm (11-9MHz). All images of the popliteal artery were acquired proximal to the branching of the tibial artery; however distance variation between participants was required to ensure the highest possible image quality. Baseline measures of the artery were recorded for 30 cardiac cycles before commencing the inflation protocol. The thigh cuff was initially inflated (E20 Rapid Cuff Inflator & AG101 Cuff Inflator Air Source, Hokanson, WA) to 90mmHg for 150 s and then deflated for 180 s. Ultrasound images of the popliteal were recorded for the final 30 s of each inflation step (after 2min of inflation) to ensure attainment of steady state flow (Takarada *et al.*, 2000b). This procedure was repeated and cuff pressure was increased incrementally by 10mmHg (150 s inflation, 180 s deflation) until 150mmHg was reached or until the popliteal flow was no longer detected.

Brachial artery. Participants moved to a supine position with their right arm extended and immobilised at an angle approximately 80° from the torso. An 11 cm wide pneumatic cuff (SC10TM segmental pressure cuff, Hokanson, WA) was positioned on the upper arm overlying marks of prior anthropometric assessment. Brachial artery diameter and velocity were measured using duplex ultrasound directly downstream of the cuff >3cm proximal of the olecranon process. With the starting pressure of 60mmHg the same inflation / deflation procedure for ultrasound image acquisition was used as described for the popliteal artery.

Analysis of blood flow restriction. Popliteal and brachial artery diameter and flow velocity during each pressure step were analysed with a custom-designed, edge detection and wall tracking software (Vascular Research Tools 5, Medical Imaging Applications, LLC, Coralville, Iowa). Media-to-media diastolic diameter was measured within a specified region of interest on B-mode images. The Doppler flow velocity spectrum was traced and TAMV (cm/s) computed. Synchronised diameter and velocity data, sampled at 20 Hz, enabled calculation of blood flow and shear rate. Resting diastolic diameter (mm) was averaged over 30 cardiac cycles. Blood flow (ml/min) was calculated as $(\text{TAMV} \times \pi r^2) \times 60$, where r is the

radius of the brachial artery lumen. Resting blood flow was averaged over 20 cardiac cycles. Shear rate was derived from Poiseuillies law and calculated accordingly as $(4 \times \text{TAMV}) / \text{diameter}$.

6.2.6 Statistical analysis

Data was analysed using SPSS statistical package (IBM SPSS Statistics 20). A Shapiro-Wilk test was used to confirm normal distribution and a Mauchley test of sphericity to verify homogeneity of variance. A two-way mixed measures ANOVA was used to evaluate the conduit artery response (diameter, blood velocity, blood flow and shear rate) to incremental pressures (popliteal 90-150 mmHg, brachial 60-120 mmHg) in males and females. Bonferroni corrected post hoc t-tests were then used to locate significance.

Blood flow at each cuff pressure was expressed relative to baseline (without cuff) to quantify the level of restriction. Individual data was then fitted with a second order polynomial equation for the prediction of cuff pressure (mmHg) at 60% BFR. Pearson's correlation was then used to determine the relationship between dependent (cuff pressure at 60% BFR) and predictor variables (anthropometry, adipose thickness, muscle CSA and blood pressure). This provided an order for entering variables into the hierarchical linear regression models. Different models of hierarchical linear regression were used to predict the pressure at which 60% BFR occurred in both the popliteal and brachial artery. Models consisted of 2-3 blocks. Pearson correlation, SEE and the change in the F value was assessed as each individual variable was added to the overall model. Only a single blood pressure variable (SBP, DBP, MAP) was entered in each model to avoid multi-collinearity.

6.3 Results

Ultrasound images were of insufficient quality in three participants preventing quantification of blood flow restriction, and were therefore excluded from the results. Whole group subject characteristics are presented in table 6.1 (n=47; 24 males, 23 females).

Table 6.1 Subject characteristics (*n*=47; *males*=24, *females*=23)

Variable	Males	Females	Combined	Minimum	Maximum
Age (years)	22 ± 3	21 ± 2	21 ± 3	18	32
Height (m)	1.81 ± 6.78	1.67 ± 6.71	1.76 ± 9.6	1.52	1.95
Mass (kg)	76.5 ± 10.1	60.8 ± 8.4	69.7 ± 12.1	46.1	94.7
BMI (kg/m ²)	23.1 ± 2.4	21.7 ± 2.7	22.5 ± 2.6	18.2	29.3
SBP (mmHg)	132 ± 11	112 ± 7	124 ± 14	96	163
DBP (mmHg)	75 ± 7	69 ± 5	72 ± 7	61	88
MAP (mmHg)	94 ± 7	84 ± 5	89 ± 8	73	106
Thigh circumference (cm)	57.9 ± 3.9	55.5 ± 4.1	57.0 ± 4.1	50.2	66.7
Thigh AT (mm)	9.8 ± 3.9	16.5 ± 4.5	12.8 ± 5.3	3.2	27.0
Thigh muscle-bone CSA (cm ²)	214 ± 30	163 ± 22	193 ± 37	131	275
Arm circumference (cm)	33.4 ± 3.0	29.5 ± 2.8	31.6 ± 3.5	24.6	38.7
Arm AT (mm)	6.4 ± 3.3	10.3 ± 4.0	8.2 ± 4.1	1.9	22.1
Arm muscle-bone CSA (cm ²)	70 ± 14	43 ± 9	57 ± 18	33	102

Values are means ± SD, range (maximum - minimum) provided for combined data. BMI Body mass index; SBP systolic blood pressure, DBP diastolic blood pressure, AT adipose thickness.

6.3.1 Conduit artery response to incremental external cuff pressure

The diameter of the popliteal and brachial artery was maintained at lower cuff pressures before decreasing at the high pressures (2-way ANOVA main effect for cuff pressure, $P < 0.001$). Baseline diameter before cuff inflation was 5.23 ± 0.76 mm and 3.76 ± 0.64 mm in the popliteal and brachial arteries respectively. Female participants had significantly smaller popliteal (4.63 ± 0.39 mm vs. 5.72 ± 0.60 mm) and brachial (3.29 ± 0.48 mm vs. 4.10 ± 0.52 mm) arteries than males ($P < 0.001$). A decrease in popliteal and brachial artery diameter began with external pressures of 130 and 120 mmHg (Bonferroni post hoc, $P = 0.01$ and $P = 0.03$, respectively). This response differed between sexes (Figure 6.1), with the diameter of popliteal and brachial arteries decreasing at 120 and 110 mmHg in females and 130 and 120 mmHg in males (2-way ANOVA, pressure by sex interaction $P = 0.04$).

Baseline blood velocity and shear rate before cuff inflation was 16.0 ± 3.7 cm/s and 123 ± 27 s⁻¹ in the popliteal artery, and 17.1 ± 5.5 cm/s and 185 ± 58 s⁻¹ in the brachial artery, respectively. A decrease in blood velocity and shear rate (vs baseline) was detected in both arteries following initial cuff inflation (2-way ANOVA main effect for cuff pressure; Bonferroni post hoc $P < 0.01$). The blood velocity and shear rate response to incremental cuff pressures did not differ between sexes (2-way ANOVA, pressure by sex interaction; $P = 0.24$ and $P = 0.172$, respectively).

Blood velocity reduced significantly with each pressure increment from 80mmHg in the brachial and 100mmHg in the popliteal (Figure 6.2). Large reductions in popliteal shear rate were seen between 90-110mmHg before a more gradual decline thereafter (Figure 6.3). In contrast a steep decline in brachial artery shear rate was observed from 80mmHg (Figure 6.3).

Baseline blood flow before cuff inflation was 217 ± 88 ml/min and 119 ± 63 ml/min in the popliteal and brachial arteries, respectively. Blood flow at each pressure increment was expressed relative to baseline blood flow revealing a polynomial relationship between cuff pressure and the level of blood flow restriction (Figure 6.4)

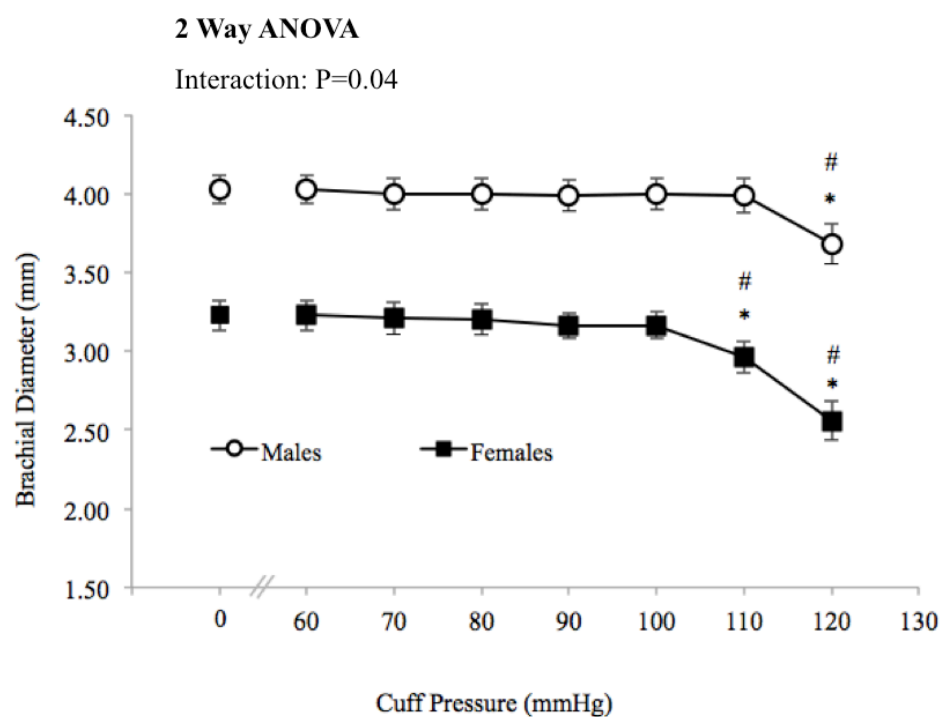
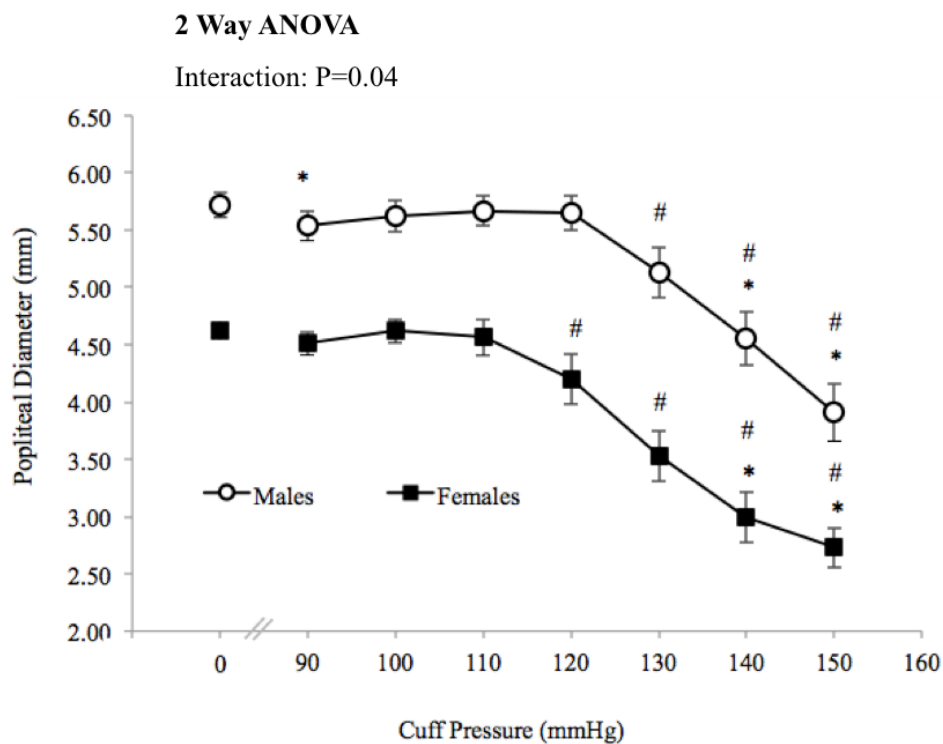


Figure 6.1 Diameter of the popliteal and brachial arteries at different external cuff pressures in males ($n=24$) and females ($n=23$). Values are means \pm SEM. Significantly different to * resting diameter and # previous cuff pressure ($P<0.05$) with Bonferroni t-test.

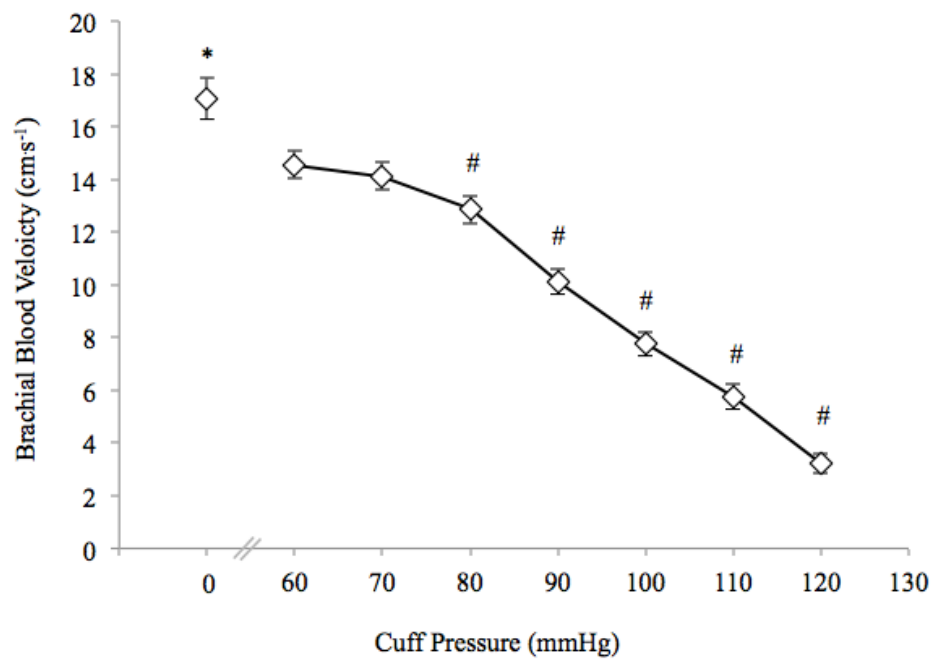
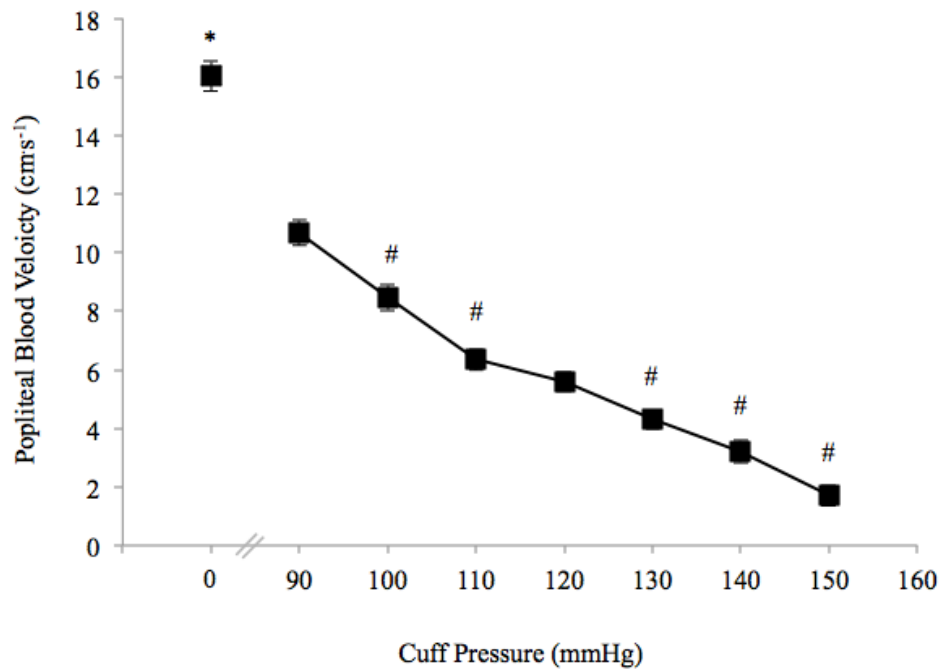


Figure 6.2 Blood velocity in the popliteal and brachial arteries at different external cuff pressures ($n=47$). Values are means \pm SEM. Significantly different to # previous and * all cuff pressures ($P<0.05$) with Bonferroni t-test.

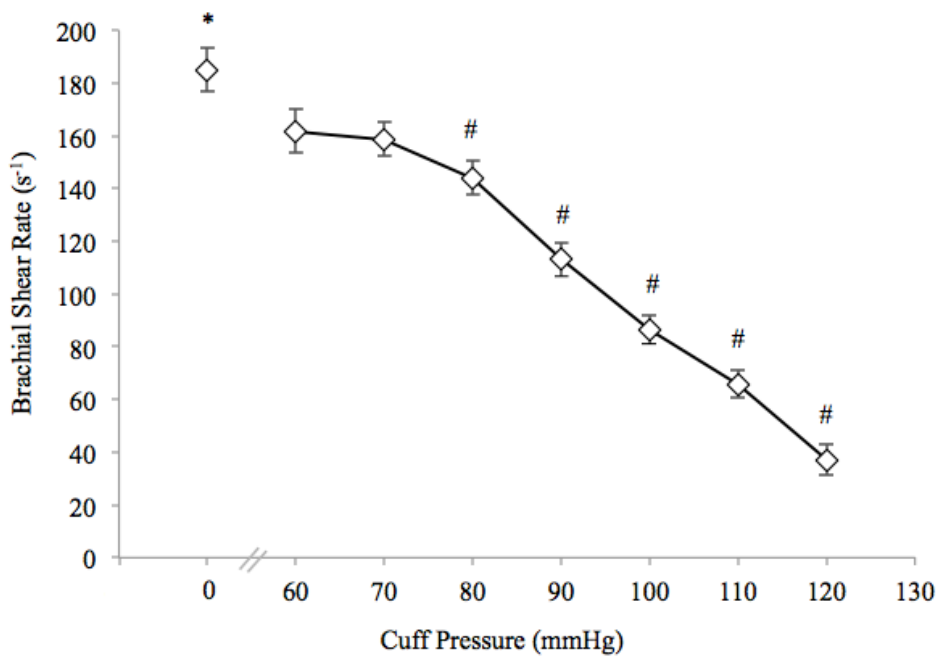
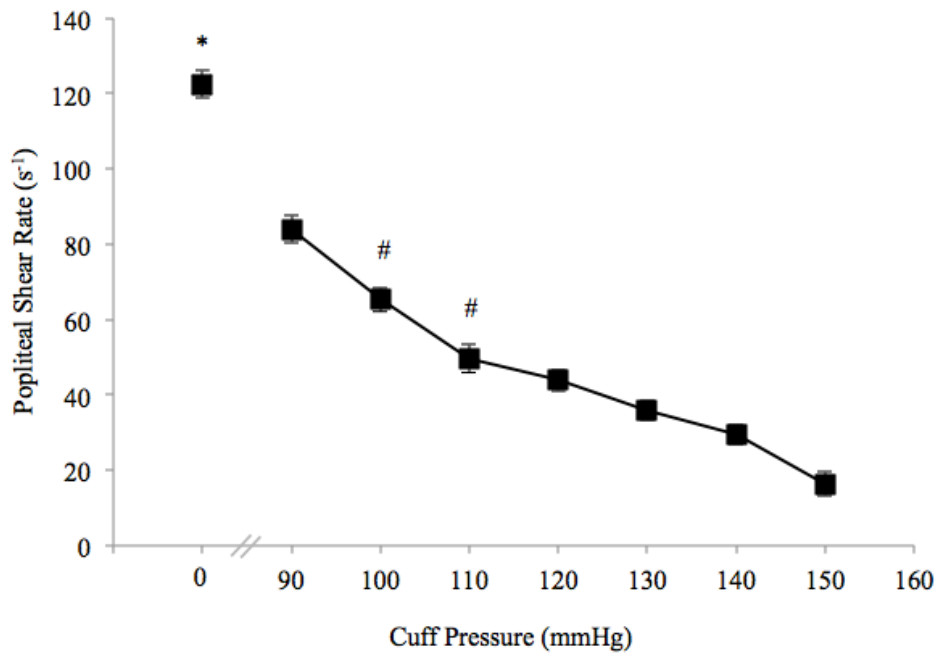


Figure 6.3 Shear rate in the popliteal and brachial arteries at different external cuff pressure ($n=47$). Values are means \pm SEM. Significantly different to # previous and * all cuff pressures ($P<0.05$) with Bonferroni t-test.

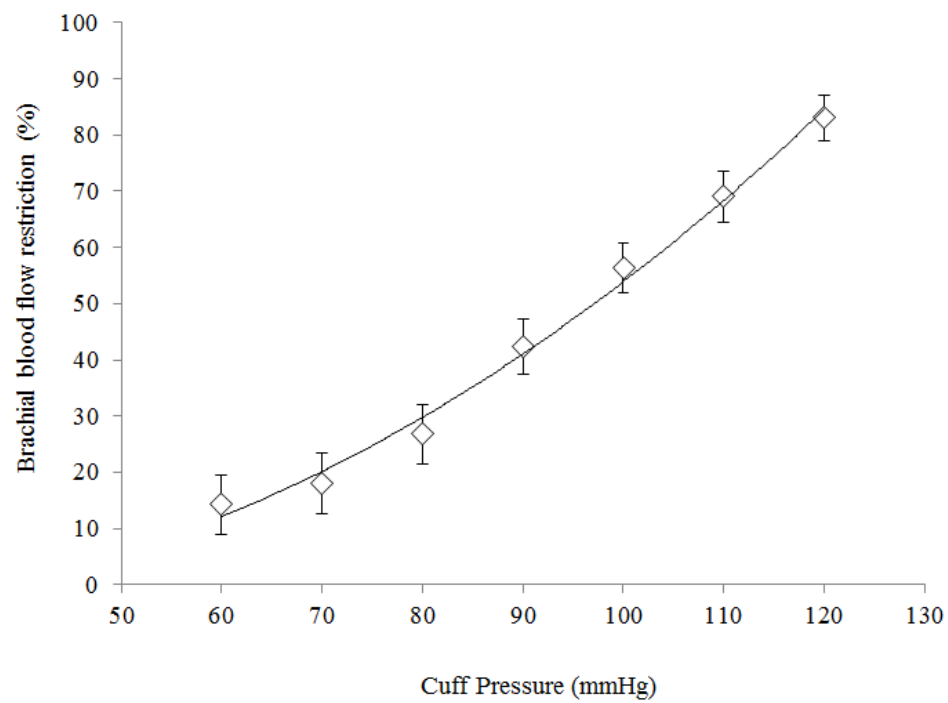
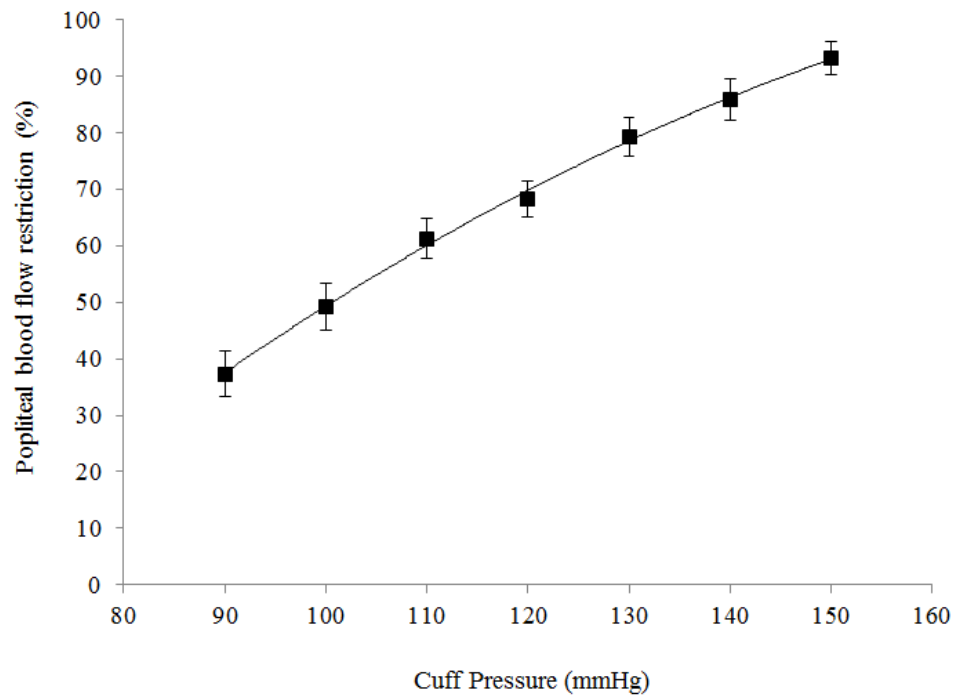


Figure 6.4 Blood flow restriction (% of resting blood flow) in the popliteal and brachial arteries with cuff pressure ($n=47$). Values are means \pm 95% confidence intervals.

6.3.2 External cuff pressure and the level of blood flow restriction

Individuals' blood flow restriction data was fitted with a second order polynomial equation for the prediction of cuff pressure at 60% BFR (Figure 6.4). Combined analysis revealed a significant difference in the cuff pressure required to elicit 60% BFR in the popliteal (111 ± 12 mmHg) and brachial arteries (101 ± 12 mmHg, $P = 0.0002$). Partial BFR (60%) was achieved in the popliteal artery at different cuff pressures for males (114 ± 15 mmHg) and females (105 ± 10 mmHg) ($P = 0.03$). In contrast, there was no difference in the pressure required to elicit 60% BFR in the brachial artery between males (102 ± 18 mmHg) and females (100 ± 14 mmHg) ($P = 0.67$). No relationship was observed between thigh and arm cuff pressures ($r = 0.02$).

Correlations between subject variables and the external cuff pressure required for 60% BFR are presented in table 6.2.

Table 6.2 Pearson correlations between subject characteristics and the external cuff pressure required for 60% blood flow restriction in the popliteal and brachial arteries

Variable	Popliteal	Brachial
BMI (kg/m ²)	0.44 *	-0.26
SBP (mmHg)	0.49 *	0.27
DBP (mmHg)	0.57 *	0.39 *
MAP (mmHg)	0.58 *	0.38 *
Thigh circumference (cm)	0.34 *	
Thigh AT (mm)	-0.03	
Thigh muscle-bone CSA (cm ²)	0.31 *	
Arm circumference (cm)		-0.23
Arm AT (mm)		-0.45 *
Arm muscle-bone CSA (cm ²)		0.07

Note: BMI body mass index, SBP systolic blood pressure, DBP diastolic blood pressure, AT adipose thickness

Popliteal artery models. Variables were entered into the first hierarchical regression models for the popliteal in order of correlation strength. MAP had the strongest relationship with cuff pressure required for 60% BFR ($r = 0.58$, Table 6.2) independently explaining 34% of the variance. Entry of BMI into the regression model (block 2 of model 1 [Table 6.3]) explained

an additional 10% variance (total $r^2 = 45\%$) in the cuff pressure required for 60% BFR, but adding thigh circumference (block 3 of model 1) did not (Sig F change = 0.457, Table 6.3).

Block 2 of model 2 (Table 6.4), composed of DBP and BMI, explained the most variance ($r^2 = 48\%$) in the cuff pressure required for 60% BFR. The addition of thigh circumference did not explain any additional variance (Sig F change = 0.936). Standardised betas and part correlation coefficients indicated that DBP explained most variance from each individual block. A reduced SEE indicates an increased accuracy of prediction with model 2 compared to model 1 (9.06 vs. 8.83 respectively).

To exclude potential error associated with BMI in certain populations (athletes), a third model (Table 6.5) was composed of DBP and thigh circumference, which explained 41% of the variance in the cuff pressure required for 60% BFR. The regression equations generated from model 2 and 3 were then compared using mean values (Table 6.6). The error in prediction, as indicated by 95% confidence intervals, is smaller using model 2 with DBP and BMI as predictor variables. With a DBP of 72 mmHg and BMI of 22.5 kg/m^2 , the predicted cuff pressure required for 60% BFR would fall between 103 to 119 mmHg with approximately 68% accuracy or 94 to 128 mmHg with approximately 95% accuracy.

Table 6.3 Model 1 stepwise multiple linear regression model for popliteal BFR ($n=47$)

Block 1	B	SE B	Stand β	R	SEE	Sig F change
Constant	34.83	15.87				
MAP	0.85	0.18	.58*	.58	9.75	0.001
Block 2						
Constant	11.02	16.99				
MAP	0.74	0.17	.51*			
BMI	1.49	0.53	.33*	.67	9.06	0.007
Block 3						
Constant	21.48	22.04				
MAP	0.76	0.17	.52*			
BMI	1.99	0.85	.44*			
Thigh Circumference	-0.41	0.54	-.14	.67	9.11	0.457

Note $R^2 = 0.34$ Block 1, $\Delta R^2 = 0.102$ Block 2, $\Delta R^2 = 0.00$ Block 3. * $P < 0.05$.

Table 6.4. Model 2 stepwise multiple linear regression model for popliteal BFR (n=47)

Block 1	B	SE B	Stand β	R	SEE	Sig F Change
Constant	39.62	15.58				
DBP	0.99	0.21	.57*	.57	9.89	0.001
Block 2						
Constant	4.65	17.07				
DBP	0.92	0.19	.53*			
BMI	1.78	0.50	.39*	.69	8.83	0.001
Block 3						
Constant	5.88	23.16				
DBP	0.92	0.19	.53*			
BMI	1.83	0.84	.40*			
Thigh Circumference	-0.04	0.53	-.15	.69	8.93	0.936
Note $R^2 = 0.32$ Block 1, $\Delta R^2 = 0.092$ Block 2 ($P < 0.05$), $\Delta R^2 = 0.00$ Block 3 ($P = 0.937$). * $P < 0.05$						

Table 6.5 Model 3 stepwise multiple linear regression model for popliteal BFR (n=47)

Block 1	B	SE B	Stand β	R	SEE	Sig F Change
Constant	39.63	15.59				
DBP	0.99	0.21	.57*	.57	9.90	0.001
Block 2						
Constant	-7.71	23.24				
DBP	0.95	0.20	.55*			
Thigh Circumference	0.87	0.33	.30*	.64	9.31	0.012
Note $R^2 = 0.32$ Block 1, $\Delta R^2 = 0.092$. * $P < 0.05$						

Table 6.6 Regression equations for popliteal BFR models using mean values

	Model 2	Model 3
Regression Equation	0.92(DBP) + 1.78(BMI) + 4.65 (± 8.83)	0.95(DBP) + 0.87(ThighCir) -7.71 (± 9.31)
Pressure at 60% BFR (mmHg)	111	110
95% CI (mmHg)	94 - 128	92 - 128

Note: BFR blood flow restriction, CI confidence intervals, BMI body mass index, DBP diastolic blood pressure, ThighCir thigh circumference.

Brachial artery models Variables were entered into a hierarchical regression model for the brachial in order of correlation strength. Arm AT had the strongest relationship with cuff pressure required for 60% BFR ($r = -0.45$, Table 6.2) independently explaining 20% of the variance. Entry of DBP into the regression model (block 2 of model 1 [Table 6.7]) explained an additional 9.6% variance (total $r^2 = 30\%$) in the cuff pressure required for 60% BFR. Although MAP was an independent predicting variable ($r = 0.38$, Table 6.2) entry into the model was excluded to avoid multi-collinearity. No correlation was observed between further variables (body mass, BMI, SBP, arm circumference, muscle-bone CSA) and the cuff pressure required for 60% BFR. Mean values for arm AT (8.1 mm) and DBP (72 mmHg) were entered into the regression equation generated from model 1 (Table 6.8). The predicted cuff pressure required for 60% BFR will fall between 93 to 109 mmHg with approximately 68% accuracy or 86 to 116 mmHg with approximately 95% accuracy.

Table 6.7 Model 1 stepwise multiple linear regression model for Brachial BFR ($n=47$)

Block 1	B	SE B	Stand β	R	SEE	Sig F Change
Constant	108.86	2.64				
Arm AT	-0.967	0.28	-.45	.45	7.97	0.001
Block 2						
Constant	77.49	13.01				
Arm AT	-0.83	0.27	-.39			
DBP	0.42	0.17	.32	.55	7.56	0.018

Note $R^2 = 0.206$ Block 1, $\Delta R^2 = 0.096$ Block 2, * $P < 0.05$

Table 6.8 Regression equation for brachial BFR model using mean values

	Model 1
Regression Equation	$0.42(\text{DBP}) - 0.83(\text{AT}) + 77.49 (\pm 7.56)$
Pressure at 60% BFR (mmHg)	101
95% CI (mmHg)	86 – 116

Note: BFR blood flow restriction, CI confidence intervals, DBP diastolic blood pressure, AT adipose thickness.

6.4 Discussion

This study has demonstrated between-subject variation in the level of blood flow restriction at different external cuff pressures applied to upper and lower limbs. With wide cuff application the external pressure required to illicit 60% BFR in the popliteal was 111 ± 12 mmHg and was greater than in the brachial at 101 ± 12 mmHg. MAP and AT were the largest independent determinants of lower and upper body partial occlusion (60%BFR) pressures, respectively. Despite entering further variables into an upper (DBP & BMI) and lower (DBP) limb regression model, the majority of variance remains unexplained.

Efficacy in BFR exercise is achieved by balancing the level of blood flow restriction, increased muscle activation and fatigue (contractile/metabolic impairment), so as to safeguard training load (total work done) (Yasuda *et al.*, 2008; Fahs *et al.*, 2012b). The level of BFR will influence hemodynamic signals, oxygen and nutrient delivery, and the accumulation and clearance rate of local metabolic by-products during exercise (Takarada *et al.*, 2000b; Karabulut *et al.*, 2011). This is likely to have implications on strength and vascular adaptations to BFR training, since muscle mass increases in proportion to metabolic stress under ischemic conditions (Takada *et al.*, 2012), while conduit and resistance vessel adaptation is related to the magnitude and pattern of shear stress (Green *et al.*, 2011), and capillary growth is proportional to metabolic activity (Adair & Montani, 2010). Ensuring an equal and appropriate level of BFR between participants is therefore important to achieve consistent gains from exercise training.

The inflation pressure required to elicit a 60% decrease in blood flow to the lower and upper extremities was investigated using Doppler ultrasound to monitor blood flow in the brachial and popliteal arteries, respectively. The decision to establish a 60% reduction in resting blood flow was based on the hemodynamic profile of narrow cuff inflation protocols, which have been shown to be effective in BFR training studies (Takano *et al.*, 2005). Previous investigations trying to establish the factors (blood pressure, limb size and composition) influencing the BFR stimulus have determined the external cuff pressure required for complete arterial occlusion (100% BFR) (Loenneke *et al.*, 2011), which does not reflect the partial restriction prescribed for this training technique. Assumptions of a linear relationship between

external cuff pressure and the level of BFR cannot be made. Indeed, the present study reports a curvilinear relationship between these two factors (Figure 6.4), which fits with previous observations by Laurentino et al (2012) that 50% BFR was achieved at 80% of the pressure required to occlude arterial flow.

Lower restrictive cuff pressures are generally utilised for upper body BFR exercise due to the smaller limb size (Fahs *et al.*, 2012b). In agreement, it was observed that the cuff pressure required to illicit 60% BFR was lower in the brachial versus the popliteal, but only by 10 mmHg. This does correspond with recommendations for prescribing narrow cuff restrictive pressures at 120% vs. 130% of SBP on the upper vs. lower limbs respectively (Takano *et al.*, 2005; Yasuda *et al.*, 2008). However findings equate to 81% and 90% SBP (based on participants average SBP, Table 6.1) in the present study, using wide cuffs on the upper and lower limbs. Nevertheless, the poor relationship between SBP and pressure at 60% BFR in the popliteal ($r = 0.49$) and brachial ($r = 0.27$) arteries suggests restrictive cuff pressures should not be based on SBP alone. The restriction of brachial artery blood flow at low-moderate external cuff pressures (60-100 mmHg) was less than anticipated. Previous investigations have shown increased electromyographic activity during elbow flexion exercise at cuff pressures as low as 50 mmHg (Takarada *et al.*, 2000b). In the absence of ischemia the suppressed clearance of metabolites due to venous occlusion may have increased voluntary muscle activation. This highlights the importance of considering both venous outflow and arterial inflow when quantifying the hemodynamic response to BFR exercise.

The 60% reduction in popliteal blood flow was achieved at lower external cuff pressures in females (105 ± 10 mmHg) compared to males (114 ± 15 mmHg). However, there was no difference in the pressure at 60% BFR in the brachial artery between sexes (100 ± 14 mmHg vs. 102 ± 18 mmHg). This is unlikely to be due to a disparity in limb size, since circumference of the arm and thigh are proportionately larger in males versus females. An alternative is that findings reflect differences between sexes in local vascular function. Indeed, Nishiyama et al (2008) noted that women have similar vascular function to men in the upper extremities but appear to have impaired vascular function, when normalized for shear rate, in the lower extremities. Heterogeneity of endothelial function throughout the arterial tree may account for

the absent relationship between the cuff pressure at 60% BFR in the brachial versus the popliteal artery.

Stepwise regression models were used to determine what subject characteristics should be accounted for when prescribing the restriction cuff pressure for lower and upper limb BFR training. Although MAP had the strongest independent relationship with pressure at 60% BFR in the popliteal artery, more variance (48%) was explained by entering DBP alongside BMI in the regression model. Accounting for thigh circumference and DBP, in accordance with Loenneke et al (2011), resulted in greater error in the prediction of the pressure required for 60% popliteal BFR. Conversely, AT had the strongest independent relationship with pressure at 60% BFR in the brachial artery, with DBP explaining additional variance (total 30%). In contrast to previous conceptions, arm circumference did not influence the cuff pressure required for 60% BFR in the brachial artery and was therefore not included in the regression model.

It was found that SBP, though often used to determine the restrictive cuff pressure during BFR exercise, did not explain additional variance in either lower or upper body regression models. This is in agreement with Loenneke et al (2012) and others who report a weak correlation between SBP and arterial occlusion pressure in normotensive adults (Moore *et al.*, 1987; Crenshaw *et al.*, 1988). In contrast, DBP appears a significant predictor of partial occlusion pressures, corresponding with its association with peripheral resistance (Levick, 2011).

In contrast to previous investigations (Loenneke *et al.*, 2011) a weak ($r = 0.34$) and even absent ($r = -0.23$, $P > 0.05$) relationship between limb circumference and partial occlusion pressure in the popliteal and brachial arteries was observed. This was unexpected since the percentage of external cuff pressure reflected in the underlying tissue, and therefore vasculature, is inversely related to the circumference of the limb (Shaw & Murray, 1982). However, Crenshaw et al (1988) did note arterial occlusion pressure was much more dependent on thigh circumference when using narrow as opposed to wide cuffs, as used in the present investigation.

Nevertheless, the regressions are likely constrained by the narrow variance in limb circumference (thigh 50 – 67 cm; arm 25 – 39 cm). Loenneke et al (2011) reported a larger

range in this predictor variable (thigh circumference 56-78 cm) and, perhaps as a consequence, a stronger correlation between thigh circumference and arterial occlusion pressure. Alternatively, limb circumference may not influence partial (60% BFR) as opposed to complete arterial occlusion measured previously (Loenneke *et al.*, 2011). Other factors such as local vascular function may play a more predominant role in partial occlusion pressures.

Recently, the effect of limb composition on the level of blood flow restriction has been postulated (Karabulut *et al.*, 2011). Tissue oxygenation during low level cuff inflation was negatively corrected with leg lean body mass (Karabulut *et al.*, 2011) suggesting muscle may transmit more pressure on the underlying vasculature. However, Loenneke *et al.* (2011) noted a positive relationship between muscle CSA and arterial occlusion pressure, suggesting otherwise. The present study finds a negative relationship between adipose tissue thickness and pressure at 60% BFR in the brachial artery. Further research regarding the effect of tissue composition on the level of blood flow restriction using more sensitive measures of fat and muscle mass is warranted.

The regression models formed in the present investigation account for 48% and 30% of the variance in lower and upper limb partial occlusion, but a large percentage remains unexplained. Variance may lie with individual differences in vessel characteristics. Our utilisation of duplex-Doppler ultrasound, as opposed to pulse-Doppler, allowed for insightful measures of conduit artery diameter and blood flow velocity in response to proximal cuff inflation. Conduit artery diameter was maintained at lower cuff pressures (< 120 mmHg) before rapidly decreasing. This response is delayed in males, which is consistent with evidence of a more pronounced low-flow-mediated vasoconstriction in women (Levenson *et al.*, 2001). In contrast to diameter we report a linear decline in blood velocity with increased external cuff pressure. The steep then gradual decline in popliteal shear rate is due to the limited change in diameter at lower cuff pressures followed by vasoconstriction which augments shear rate. This response was not observed in the brachial largely due to a lack of vasoconstriction over a range of lower external cuff pressures (60-120 mmHg).

An important observation is that the conduit artery does not constrict to attain a 60% reduction in blood flow. The latter is instead due to a marked decrease in blood flow velocity (time average mean velocity). Constriction of the artery in response to low-flow is mediated by

endothelin-1 (Spieker *et al.*, 2003) or through inhibiting EDHF and cyclooxygenase products (e.g. prostaglandins) (Gori *et al.*, 2008). Repeated exposure to such stimuli could result in endothelial dysfunction. BFR training studies applying higher external cuff pressures should consider the level of artery constriction and potential for functional maladaptation of the vasculature.

6.4.1 Limitations

We acknowledge the limitations of the present study. Participants were resting in a supine position during blood flow measures, while BFR protocols involve exercise and are typically performed in the seated or standing position. A similar relationship between cuff pressure and blood flow is expected, but values would be elevated due to the activated skeletal muscle pump and increased BP in response to postural changes and the pressor response of exercise. Therefore greater external cuff pressure may be required to elicit the same partial occlusion (60% BFR) stimulus. Furthermore, due to software limitations this study was unable to quantify the antegrade/retrograde pattern of blood flow, which would provide a better indicator of the shear stress stimulus elicited during BFR exercise.

6.4.2 Conclusion

This study used regression models to determine the impact of subject characteristics (BP, limb size and composition) on the external pressure (wide cuff) required to elicit 60% reduction in popliteal and brachial artery blood flow. It was found that DBP is the most important variable for the prescription of partial occlusion pressure on both the upper and lower limbs. In contrast, limb circumference has limited impact on the cuff pressure required for partial blood flow restriction. However, this may be due to the narrow variance in limb circumference observed in healthy subject populations as investigated here. Nevertheless, the majority of the variance in partial occlusion pressure remains unexplained by the predictor variables assessed in the present study. Further investigation of the influence of local vessel characteristics is

required if studies are to achieve an equal BFR between subjects. Sex sensitivities in the regulation of arterial vascular tone may also need to be considered.

Chapter 7: General discussion

7.1 Key Findings

This thesis investigated the acute response and chronic adaptations of the peripheral vasculature to low load resistance exercise with BFR. The methodology utilised Doppler ultrasound, strain gauge plethysmography and muscle biopsy for insightful measures at different regions of the vascular tree. Chapter 3 assessed brachial artery modifications to BFR handgrip training and detraining. To establish the time course of adaptation, chapter 4 examined longitudinal changes at the conduit, resistance and capillary level of the vascular tree in response to unilateral plantar flexion training with BFR. Enhanced capillarity was identified and the stimulus investigated further in chapter 5 by determining the acute angiogenic transcriptional response to low load knee extension exercise with BFR. Finally chapter 6 measured acute changes in conduit artery diameter and flow in response to varying restrictive cuff pressures to determine the variation in the relative BFR stimulus between individuals. The main findings of these studies are summarised below.

1. Low load resistance exercise with BFR causes vascular remodelling at the conduit (chapters 3, 4) resistance (chapter 4) and capillary (chapter 4) level of the vascular tree. Regional, rather than systemic, factors are responsible for these adaptations as evidenced by an absent response in the contralateral control limb.
2. In the conduit artery functional changes precede structural remodelling (chapter 4) and structural normalisation occurs following stimulus removal due to detraining (chapter 3).
3. Blood flow restriction potentiates the expression of VEGF, PGC-1 α and eNOS mRNA in response to low load resistance exercise, while up-regulating VEGFR-2 and HIF-1 α mRNA, suggesting a targeted angiogenic response potentially mediated through enhanced metabolic, ischemic and shear stress stimuli (chapter 5).
4. Large between subject variability in the level of BFR (chapter 6) may explain the variance in the acute response (chapter 5) and chronic adaptation (chapter 4) to this

mode of training. Variability in the level of lower limb BFR is mostly explained by participants DBP and BMI (Chapter 6).

7.2 Vascular remodelling and low load resistance exercise with BFR

7.2.1 Conduit arteries

The measure of DC was performed in both training studies (chapter 3 and 4) in order to examine conduit artery structure. Maximal dilation after ischemic exercise is an accepted (Naylor *et al.*, 2005) and frequently used method for determining conduit artery remodelling in response to training (Tinken *et al.*, 2008, 2010). This thesis reports a 3.1% and 3.3% increase in brachial and popliteal maximal diameter (elicited by the DC ischemic exercise stimulus) following 4 and 6 weeks low load resistance exercise with BFR, respectively. This demonstrates a similar adaptive response of conduit arteries in the upper and lower limbs as observed previously (Tinken *et al.*, 2008).

The changes are larger than the axial resolution limit of our high frequency transducers (11MHz) and greater than 95% confidence intervals (0.06mm brachial & 0.13mm popliteal) for the parameter of maximal diameter, suggesting a significant training effect. However, remodelling of a greater magnitude is found over the same time course following localised non-BFR exercise (Tinken *et al.*, 2010) perhaps due to the higher training load (30 min at 30–50% MVC, 4 d/wk) compared to BFR protocols (5-10 min at 30-40% 1RM, 3 d/wk). The recruitment of habitually active participants in the present thesis possibly reduced the potential for adaptation (Kojda & Hambrecht, 2005), with previous studies finding a modest increase (3.8%) in maximal brachial diameter in already trained individuals (Naylor *et al.*, 2006). Furthermore, studies recruiting untrained and sedentary populations report a greater increase in conduit artery diameter with exercise training (Dinenno *et al.*, 2001; Zoeller *et al.*, 2009). Changes in conduit artery structure in response to BFR training occur over a similar time course as previously demonstrated. For example, maximal diameter of the brachial and popliteal arteries increased after 4 weeks handgrip and cycling training respectively (Tinken *et al.*, 2008, 2010). Conduit arteries continued to enlarge over the remaining 4 weeks of the 8-

week intervention (Tinken *et al.*, 2008, 2010) suggesting arterial remodelling of a greater magnitude may have occurred in our studies with longer BFR training protocols.

The process of conduit artery adaptation to exercise training was originally identified in animal models (Laughlin, 1995) and later confirmed in human studies (Tinken *et al.*, 2008). This model of adaptation depicts an early increase in endothelium-mediated vasodilation before arterial remodelling normalises shear stress and removes the stimulus for further functional adaptations. Although in chapter 3 an early functional change was not observed this was likely due to the lack of regular measures, which meant the response was missed. To deduce if functional adaptations precede structural modification in response to low load resistance exercise with BFR, the time course of conduit artery changes was determined in chapter 4. Measures of FMD and DC were performed every 2 weeks in an attempt to track the changes in function and structure during BFR planar flexion training. The observed increase in conduit FMD by week 2 is consistent with previous investigations (Tinken *et al.*, 2008, 2010; Birk *et al.*, 2012) and suggests the BFR stimulus augments the ‘intensity and metabolic stress’ associated with low load resistance exercise, meeting the threshold for improvement in endothelial function (Goto *et al.*, 2003). The decline in FMD over the succeeding weeks has been observed previously (Tinken *et al.*, 2008; Birk *et al.*, 2012) and is superseded by a significant increase in popliteal maximal diameter at week 6, which is consistent with the hypothesis that functional vascular adaptations precede arterial remodelling.

7.2.2 Resistance vessels

Resting blood flow was unaffected by localised exercise training in agreement with others (Green *et al.*, 1994; Alomari & Welsch, 2007; Tinken *et al.*, 2008; Alomari *et al.*, 2010). The 24% and 29% improvement in peak reactive hyperaemic blood flow after 4 weeks planter flexion (chapter 4) and handgrip training (chapter 3), respectively, is consistent with early reports using this BFR training stimulus (Patterson & Ferguson, 2010) and compares favourably with heavier load non-BFR exercise (Alomari & Welsch, 2007; Alomari *et al.*, 2010).

Reactive hyperaemia describes the transient increase in blood flow after a period of limb ischemia. The magnitude of this response is governed by downstream resistance vessel CSA and is therefore used to inform the impact of exercise training on resistance vessel structure (Thijssen *et al.*, 2010). However, this is based on the assumption of eliciting a maximum vasodilatory response with diminished impact from vessel functional characteristics. In this thesis, the reactive hyperaemic response was measured using strain-gauge plethysmography (chapter 3) and Doppler ultrasound (chapter 4) following 5-min ischemia and ischemic exercise, respectively. Due to the short duration of ischemia (5-min), the protocol adopted in chapter 3 is unlikely to have elicited maximal hyperaemic blood flow (Sinoway *et al.*, 1986; Naylor *et al.*, 2005). As a result myogenic and metabolic autoregulation may have contributed to the reactive hyperaemic response independent of structural adaptations (Joyner *et al.*, 2001). In contrast, peak reactive hyperaemic blood flow is induced in response to ischemic exercise (Naylor *et al.*, 2005) validating the index of resistance vessel structure in chapter 4. An enhanced total resistance vessel CSA may have occurred from increased density and diameter of smaller (<40 μm in diameter) and larger resistance vessels respectively (White *et al.*, 1998).

The time course of resistance vessel adaptation in response to BFR planter flexion training (chapter 4) appears in line with observations during non-BFR rhythmic handgrip training (Green *et al.*, 1994; Tinken *et al.*, 2010; Alomari *et al.*, 2010). Trends for 20% increase in peak blood flow after 2 weeks imply a rapid structural remodelling of the resistance vessels. This is consistent with evidence that reactive hyperaemia, which is affected by vessel functional characteristics (Meredith *et al.*, 1996), is increased after just 1 week of localised exercise (Alomari & Welsch, 2007; Alomari *et al.*, 2010), in keeping with the concept that changes in function precede structural adaptation. To ratify the time course of resistance vessel adaptation would require weekly longitudinal measures of limb blood flow response to an ischemic challenge (10-min ischemia or 5-min ischemic exercise) and to intra-arterial infusion of endothelium-dependent (acetylcholine) and independent (sodium nitroprusside) vasoactive substances (Green *et al.*, 1994; Wilkinson & Webb, 2001). This would confirm the nature of resistance vessel adaptation as functional, structural or both.

7.2.3 Capillaries

Plethysmography measures of capillary K_f provide an index of capillarity. This is based on the strong relationship between total capillary surface area and K_f (Gamble *et al.*, 2000). It is further endorsed by the correlation between changes in K_f and direct measures of the length of capillary-to-fibre contact following endurance training (Charles *et al.*, 2006). In response to 6 week localised BFR plantar flexion training K_f was increased by 14% (chapter 4). Although this is less than has been reported previously (Evans *et al.*, 2010) our BFR protocol utilised a lower training load (intensity & volume) as well as the application of continuous occlusion.

An increase in the capillary network is a recognised adaptation with skeletal muscle hypertrophy through strength training. Indeed, capillary changes have been shown to be proportional to muscle fibre growth (McCall *et al.*, 1996), suggesting that the two processes are initiated and/or driven by some common factors (Plyley *et al.*, 1998). As a result, an increase in capillarisation is not typically observed until after approximately 12 weeks of heavy load resistance exercise training (McCall *et al.*, 1996; Green *et al.*, 1999). Although unreported in this thesis, rapid skeletal muscle hypertrophy is observed in response to BFR resistance exercise (Abe *et al.*, 2005) and consequently capillarisation follows suit. Evans *et al.* (2010) demonstrated an increase in K_f after 4 weeks of low intensity resistance-type exercise with BFR. In contrast Brown *et al.* (2001) observed no change in K_f following 4 weeks high intensity plantar flexion training (70% peak power, 20 min-day, 5 d/wk). Rapid capillarisation is reported in animal models (Hudlicka, 1998; Suzuki *et al.*, 2001). In humans a higher number of proliferating ECs is observed after 2 weeks of prolonged passive movement exercise (90 min-day, 4 d/wk) before capillary density is increased at 4 weeks (Høier *et al.*, 2010). Similarly, capillary-to-fibre ratio was unchanged after 2 weeks but evident after 4 weeks of high intensity intermittent endurance exercise (60 min-day, 5 d/wk) (Jensen *et al.*, 2004). Ours and previous findings (Evans *et al.*, 2010) would suggest BFR resistance exercise can, therefore, effectively increase muscle capillarity even though the exercise is performed at low loads and lasts for only a few minutes. This is consistent with the augmented capillarisation response to strength training with vascular occlusion in rat calf muscle (Suzuki *et al.*, 2000).

The data presented in chapters 3 & 4 demonstrate changes at the conduit, resistance and capillary level of the vascular tree in response to low load resistance exercise with BFR. A distinct time course of vascular adaptation is observed at different locations of the vascular tree (Figure 7.1). Initial remodelling appears to occur at the distal end of the vascular tree in agreement with the current hypothesis (Jasperse & Laughlin, 2006; Birk *et al.*, 2012). This is in line with the trend of increased peak reactive hyperaemia which occurred prior to changes to popliteal artery structural capacity (chapter 4) and in light of the rapid capillarisation response to BFR resistance training previously observed (Evans *et al.*, 2010).

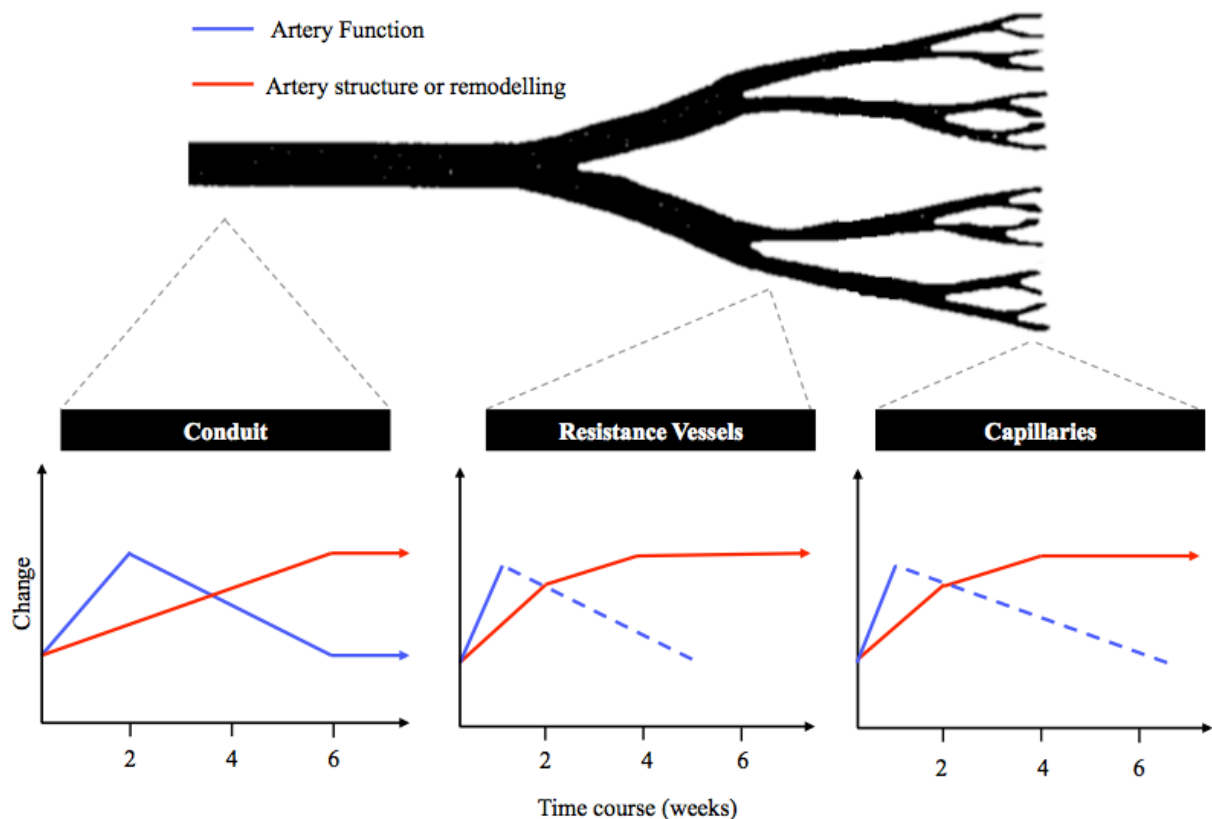


Figure 7.1 The hypothesised time course of functional and structural adaptations throughout the vascular tree in response to low load resistance exercise with blood flow restriction.

7.2.4 Vascular remodelling and deconditioning

In chapter 3, measures of FMD, DC and reactive hyperaemia were repeated two weeks after the cessation of BFR handgrip training. Whilst FMD remained unchanged, both maximal brachial artery diameter and forearm peak post-occlusion blood flow returned to baseline values after this two-week detraining period. Similarly, Stebbings et al (2013) noted femoral artery diameter had returned to baseline just 2 weeks after cessation of 8 weeks heavy load resistance training while Alomari et al (2010) observed a rapid return of forearm reactive hyperaemic blood flow to pre-training levels 1 week after the cessation of 4 weeks handgrip training. Inward remodelling of the resistance vessels occurs after just eight days of forearm physical inactivity (arm sling) before changes in maximal brachial diameter are observed (Birk *et al.*, 2013). Whilst it appears the vasculature adapts rapidly following removal of the BFR training stimulus, due to limited repeated measures we are unable to confirm if there is a distinct time-course for vascular remodelling between resistance and conduit arteries, as observed in the aforementioned study.

7.3 Mediators of vascular adaptations to low load resistance exercise with BFR

The physiological stimuli which mediate vascular adaptation are chemical signals (e.g. hypoxia, metabolites released from the muscle, cytokines) and haemodynamic mechanical signals acting on the endothelium (e.g. shear and circumferential stress) (Whyte & Laughlin, 2010). Arterial remodelling and angiogenesis are processes of vascular growth that occur in different portions of vascular tree and are primarily regulated by different stimuli (Yang *et al.*, 2008). Hemodynamic signals initiate improvements in vascular function followed by structural enlargement (arterial remodelling) in the conduit and resistance vessels. Chemical signals activated by local tissue hypoxia and metabolic activity stimulate the formation of new capillaries from pre-existing vessels (angiogenesis). BFR exercise disturbs hemodynamic and metabolic signals and expands the ischemic vascular territories, foreseeably influencing blood vessel physiology. The focus of this thesis was to report such regional vascular adaptations

specifically in response to BFR training (chapter 3 & 4) rather than explain the underlying mechanisms. Therefore, this thesis can only speculate the physiological mediators of regional vascular adaptation to low load resistance exercise with BFR. This discussion is supplemented by highlighting the acute changes in the skeletal muscle gene expression (chapter 5), which may inform the physiological signals.

7.3.1 Conduit arteries

Blood vessel walls are subjected to multiple mechanical forces caused by blood flow (shear stress), blood pressure (circumferential stress) and vasodilation (circumferential stretch) (Laughlin *et al.*, 2008). ECs form the inner layer of the blood vessels and are therefore the most important cell type exposed to these mechanical forces. The EC senses these stimuli and respond by modifying intracellular signalling, gene expression and protein expression to result in functional regulations (cell migration, remodelling, proliferation, regulation of VSM contractility) and consequently vascular adaptation (Chien, 2007). Elevated shear stress on the endothelial surface resulting from increased blood flow is considered the primary hemodynamic stimulus that prompts conduit artery adaptation (Tinken *et al.*, 2010; Naylor *et al.*, 2011). Shear stress is sensed by the endothelial glycocalyx which activates the PI3 kinase-PKB pathway and induces Akt-mediated phosphorylation of eNOS at serine 1177 & 1179 (Sessa, 2004; Michel & Vanhoutte, 2010). This alters the sensitivity of eNOS to calcium increasing its activation (Kojda & Hambrecht, 2005). Repeated exposure to increase shear stress during bouts of exercise alter EC phenotype, up-regulating eNOS protein expression (and phosphorylation status), thereby improving NO bioavailability and endothelial function (Hambrecht *et al.*, 2003; Laughlin *et al.*, 2008).

Shear stress sensed by the ECs initiates the necessary NO dependent signals for arterial enlargement involving the remodelling of the extracellular matrix. Arterial expansion continues until shear stress has been restored to baseline (Tuttle *et al.*, 2001). More recently Tinken *et al.* (2010) demonstrated the pivotal role of shear stress in determining conduit artery adaptation to localised exercise training in humans. Applying a distal forearm cuff during handgrip training attenuated exercise induced shear rate (by >50%) and prevented functional

change (FMD) and structural enlargement (DC) of the brachial artery. It was later confirmed that repeated increases in blood flow independent of exercise (via forearm heating) could also enhance brachial artery vasodilator function (FMD), reinforcing the importance of the direct impact of shear stress (Naylor *et al.*, 2011). These studies provide compelling evidence to suggest the magnitude of the shear stress stimulus induced by exercise impacts conduit artery adaptations to training.

In chapter 6 attempts were made to determine the shear stimulus elicited during upper (chapter 3) and lower limb (chapter 4) BFR protocols. Males, on average, experienced a 23% decrease in brachial artery shear rate with upper arm cuff inflation at 80 mmHg. Although performing handgrip exercise during a similar cuff inflation protocol is shown to induce a 2-fold increase in antegrade flow, a significant retrograde component remains (Credeur *et al.*, 2010). This suggests an oscillatory shear profile was induced downstream of the cuff. The shear stress stimulus would have been magnified on the artery walls underlying the cuff due to a compression induced decrease in artery diameter (Bank *et al.*, 1995). Enhanced eNOS expression is reported in ECs exposed to high shear regions underlying a cast in animal models (Cheng *et al.*, 2005; Chiu & Chien, 2011). Repeated exposure to high shear stress could therefore explain conduit arterial remodelling in response to BFR handgrip training, given the close proximity between the site of cuff application during training and location of ultrasound imaging.

A greater reduction in shear rate (~58%) was experienced in the popliteal artery with thigh cuff inflation at 110 mmHg, applied for plantar flexion BFR training (chapter 4). With this greater level of occlusion, arterial inflow remains depressed during exercise as demonstrated previously (Takano *et al.*, 2005). Subsequently, the magnitude of shear stress during BFR exercise would fall short of the threshold thought necessary to induce the functional adaptations observed (Naylor *et al.*, 2011; Birk *et al.*, 2012). Again the impact of BFR exercise on flow pattern should be considered. Whilst prolonged exposure (>20-min) to disturbed flow is associated with increased ROS production, endothelial injury (Jenkins *et al.*, 2013) and dysfunction (Rakobowchuk *et al.*, 2013), short duration exposure to oscillatory flow may elicit a positive shear stimulus on the endothelium (Green *et al.*, 2005). Indeed, Green *et al.* (2005) found oscillatory blood flow patterns in the brachial artery increased eNOS

generated NO release. An increase in NO bioavailability would explain acute improvement in femoral FMD after enhanced external counter-pulsation treatment, which increases retrograde shear stress and retrograde turbulent blood flow in the femoral artery (Gurovich & Braith, 2013). It is therefore reasonable to propose that repetitive enhancement of NO production by ECs exposed to oscillatory blood flow during short duration BFR exercise protocols would lead to an up regulation of eNOS expression and improved endothelial function (Laughlin *et al.*, 2008). In addition, post-ischemic exercise hyperaemia (after cuff deflation) increases antegrade shear stress and promotes FMD of the upstream conduit vessel (Agewall *et al.*, 2002). Blood flow can remain elevated (2-fold above rest) for more than 1-hour post BFR exercise (Gundermann *et al.*, 2012) demonstrating the prolonged nature of this shear stimulus, which with repeated exposure may mediate endothelial adaptation and subsequent arterial remodelling.

Arterial remodelling is dependent on activation of ECs, the subsequent adhesion of leucocyte (monocytes and T-lymphocyte) and EPCs, and their invasion and production of growth factors in the vessel wall (Heil & Schaper, 2004; Hoefer *et al.*, 2013). This process is facilitated by an increase in cell adhesion molecules (Heil & Schaper, 2004), which can occur as a result of oscillatory shear conditions on the ECs (Chappell *et al.*, 1998; Hsiai *et al.*, 2003). This suggests that the blood flow pattern during BFR resistance exercise could activate the ECs and may play a pivotal role in the initial stages of arterial remodelling. Further investigation is required to classify this as good or bad relative to vascular health, given the strong links between structural remodelling and atherosclerosis (Newcomer *et al.*, 2011). The remodelling process may be further promoted by VEGF as it stimulates the proliferation of ECs (Semenza, 2007a), integrin expression on monocytes (Heil *et al.*, 2000) and EPC mobilisation (Semenza, 2007a). An expansion of ischemic vascular territory during BFR resistance exercise may enhance cellular VEGF production (chapter 5). This up-regulation in the VEGF signalling pathway seems to be an important mechanism for increased conduit artery diameter in response to exercise training (Lammers *et al.*, 2012) and may explain arterial remodelling following low load BFR resistance exercise.

7.3.2 Resistance vessels

As with conduit vessels, shear stress is thought to be a key stimulus for the local exercise-induced adaptation in the resistance vessels (Tuttle *et al.*, 2001; Tinken *et al.*, 2010). As such, the shear stress signals already discussed above may be responsible for the resistance vessel remodelling in response to BFR resistance exercise (chapter 3 & 4). That said, the shear stress stimulus is likely to be dampened by the increased downstream vascular resistance caused by venous occlusion and elevated venous pressure. Distension of the vessel wall due to increased intravascular pressure may instead activate ECs and VSM cell proliferation. The growth of pre-capillary arterioles (<40 μm in diameter) is thought to occur by apposition of VSM cells to newly formed capillaries, and is mediated by the stimulus of circumferential wall stress rather than increases in shear stress (Price & Skalak, 1994; Hudlicka & Brown, 2009).

The closer proximity of the resistance vessels to the active skeletal muscle means chemical factors play a more prominent role in vascular control and potentially the adaptive process. This is demonstrated by the observation that repeated increases in blood flow and shear rate induced by forearm heating (independent of exercise), enhanced conduit artery function and structure but had limited effect on an index of resistance vessel remodelling (shear rate response to ischemic exercise) (Naylor *et al.*, 2011). This is unlikely to be due to the lack of mechanical compression from skeletal muscle contraction as this stimulus has been shown to have minimal impact on peak reactive hyperaemic blood flow (Roseguini *et al.*, 2011). It could however highlight the importance of exercise-induced metabolic stimuli, which was absent with forearm heating.

There is evidence to suggest the ischemic and metabolic signals associated with BFR exercise could enhance eNOS activation and VSM vasodilation, potentially initiating functional and structural adaptations of the resistance vessels. Using P-magnetic resonance spectroscopy, Suga *et al* (2009) demonstrated an increase in high-energy phosphate metabolism during low load resistance exercise with BFR. The enhanced breakdown of ATP may stimulate AMPK, which is found to phosphorylate eNOS on serine 1177. This phosphorylation of eNOS contributes to improvement of endothelium-dependent vasodilation induced by exercise

(Hambrecht *et al.*, 2003). Chapter 5 provides evidence that AMPK signalling pathways may be activated, as downstream target PGC-1 α (mRNA) is up-regulated in response to an acute bout of low load BFR resistance exercise. Activation of PGC-1 α may explain the enhanced transcription of VEGF mRNA (Chapter 5) and increased protein levels in circulation (Takano *et al.*, 2005) following BFR resistance exercise. This may increase VEGF availability and subsequently VEGFR-2 activation on the ECs (Shen *et al.*, 1998). This process stimulates both P13K, which is essential for Akt mediated phosphorylation and activation of eNOS, and phospholipase C, which activates calmodulin, leading to efficient NO synthesis from eNOS (Sessa, 2004; Michel & Vanhoutte, 2010). Lammers *et al.* (2012) reported improvements in peak hyperaemic flow following exercise training were strongly associated with the activation of VEGF signalling pathways, suggesting it has a central role in mediating resistance vessel remodelling.

Local tissue hypoxia induced by BFR stimulates HIF pathways (chapter 5) which can increase transcription of VEGF mRNA, enhance protein levels and binding to cognate receptors (VEGFR-1/VEGFR-2) on the ECs (Semenza, 2007a). The up-regulation of HIF-2 α is associated with resistance vessel arterial remodelling in response to exercise training (Lammers *et al.*, 2012), suggesting that activation of this hypoxic signalling pathway may be involved in the remodelling response to BFR resistance exercise. Furthermore, ischemic exercise appears to be a powerful stimulus for increased EPCs (Sandri *et al.*, 2011). EPCs contribute to maintaining the integrity of the EC layer (Gielen *et al.*, 2010) and remodelling responses in the vessel wall (Semenza, 2007a; Sirker *et al.*, 2009). EPCs have the ability to home to sites of ECs activation in ischemic tissue (Zampetaki *et al.*, 2008) perhaps accounting for the localized adaptation of the BFR trained limb.

Metabolic vasodilators (NO, adenosine, ATP, K⁺, lactate, CO₂) released by the contracting muscle bind to receptors located on the VSM activating relaxation and stretch of the ECs (Korthuis, 2011). The release of NO from skeletal muscle may be enhanced during low load resistance exercise with BFR as a result of the increased transcription of nNOS (Larkin *et al.*, 2012). This may activate the NO-dependent pathways necessary for vessel enlargement, consistent with the finding that NO signalling through nNOS is associated with resistance

vessel remodelling in response to exercise training (Lammers *et al.*, 2012). Arterial remodelling of the resistance vessels is further facilitated by purinergic signalling, since ATP and its breakdown product, adenosine, regulate VSM and EC proliferation (Burnstock, 2009).

Whilst enhanced reactive hyperaemic blood flow is thought to reflect structural enlargement of the resistance vessels, this thesis cannot exclude the possibility that maximal vasodilatory capacity was influenced by altered VSM sensitivity to vasodilatory stimuli (NO, adenosine, prostaglandin, ATP) (Laughlin & McAllister, 1992; Hambrecht *et al.*, 2000). Blood flow responses to sodium nitroprusside infusion is commonly unaltered following conventional exercise training suggesting unchanged VSM sensitivity to NO (Green *et al.*, 2011b), but this requires confirmation with BFR exercise. VSM sensitivity to adenosine, prostaglandin and ATP may have been enhanced by activation of adenosine purinergic A_{2A} receptors and ATP-sensitive K⁺ channels, as this is evoked through ischemic preconditioning (Riksen *et al.*, 2004). BFR resistance exercise shares similarities with the ischemic preconditioning stimulus so potential changes in VSM sensitivity should be investigated. The sensitivity of resistance vessels to adenosine could be determined by measuring the blood flow response to intra-arterial adenosine infusion before and after BFR exercise training.

7.3.3 Capillary vessels

Angiogenesis is stimulated in response to four physiological signals; decreased oxygen tension, raised muscle metabolism, elevated shear stress and dynamic stretch of the tissue (Egginton, 2009). Whilst mechanical factors have an accessory role in angiogenesis the overall regulation is dominated by the chemical signals of hypoxia and/or metabolic stress (Adair & Montani, 2010). This is demonstrated most in patients with peripheral arterial disease, who have increased capillary density despite chronic reductions in limb blood flow (Yang *et al.*, 2008).

The BFR stimulus limits arterial inflow and occludes venous return, causing not only tissue hypoxia but also the deprivation of nutrients and the suppressed clearance of metabolic products (Kawada, 2005; Tanimoto *et al.*, 2005). This thesis utilised a continuous BFR

protocol whereby the cuff remained inflated for the duration of exercise and the intervening recovery periods. This stimulus induces a magnified and prolonged decrease in muscle oxygen levels during low load resistance exercise (Kawada, 2005; Tanimoto *et al.*, 2005; Larkin *et al.*, 2012) and is reflected by the enhanced metabolic perturbations in the working muscle (increased PCr depletion, Pi splitting, and decreased muscle pH) (Suga *et al.*, 2009) and the increased lactate acid response to BFR exercise (Takarada *et al.*, 2000a; Sato *et al.*, 2005; Takano *et al.*, 2005; Reeves *et al.*, 2006). With the utilisation of moderate loads (as adopted in chapters 3 [40%1RM] and 4 [30% 1RM]) the intramuscular stress can mimic that of heavy load/high intensity exercise without BFR (Reeves *et al.*, 2006; Krustup *et al.*, 2009; Suga *et al.*, 2010). It is primarily these signals of local tissue ischemia and/or metabolic stress that modulate the expression of growth factors, their receptors and transcriptional regulators of the angiogenesis (Figure 7.2). In support, this thesis finds an increased transcript (mRNA) expression of VEGF, VEGFR-2, HIF-1 α , PGC-1 α and eNOS following a bout of low load knee extension exercise with BFR (Chapter 5). Repeated exposure to an acute angiogenic response such as this can stimulate capillary growth (Hellsten *et al.*, 2008; Høier *et al.*, 2010; Hoier *et al.*, 2012) and may explain the enhanced K_f following BFR plantar flexion training (Chapter 4). The acute angiogenic response was absent following non-BFR control exercise (Chapter 5) consistent with notion that capillary growth is dependent on exercise intensity (Hudlicka & Brown, 2009) and not observed after lower intensity regimes.

The activation of the VEGF signalling pathway is essential for initiating angiogenesis and is common to both morphological forms of capillary growth (longitudinal splitting and sprouting) (Williams *et al.*, 2006a; Egginton, 2011). Results from chapter 5 suggest BFR resistance exercise is capable of activating the VEGF signalling pathway in spite of low workloads (20% 1RM). BFR was found to potentiate the transcript expression of VEGF in response to low load bilateral knee extension exercise, in agreement with previous observations (Larkin *et al.*, 2012). The concurrent up-regulation of VEGFR-2 mRNA suggest VEGF activity was targeted on the ECs (Shen *et al.*, 1998). VEGF activation of the VEGFR-2 is crucial in this process because exercise-induced angiogenesis is abolished in skeletal muscle in which the receptor is inhibited (Lloyd *et al.*, 2005; Milkiewicz *et al.*, 2005). If changes in VEGFR-2 mRNA are followed by increased protein expression this could enhance tissue

responsiveness to VEGF, amplify the angiogenic cascade and capillarisation response (Gustafsson *et al.*, 2007).

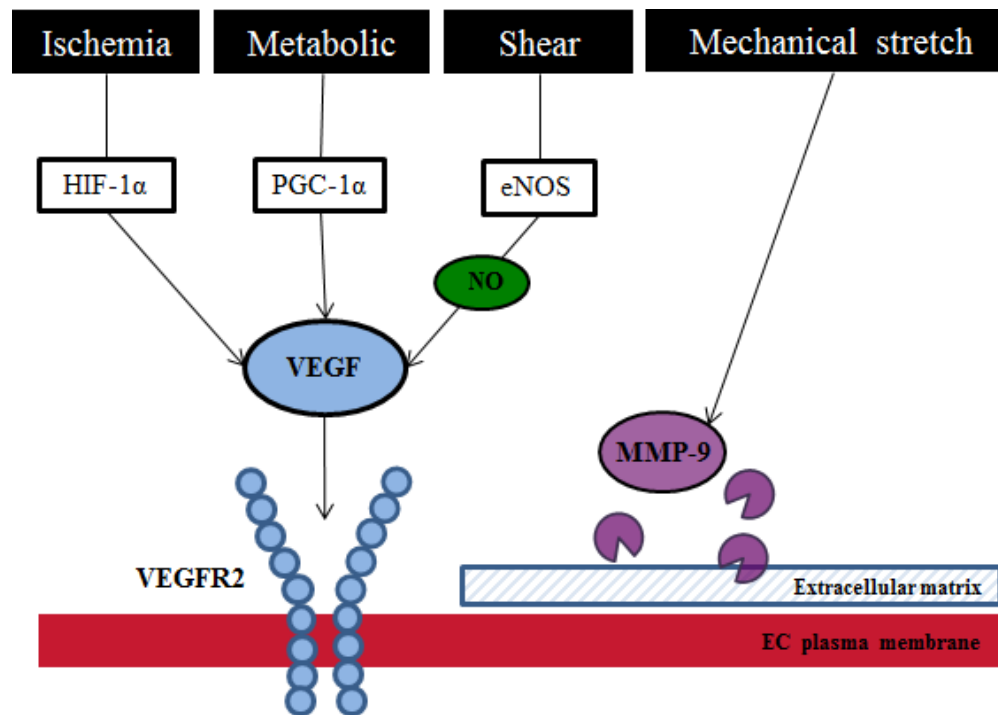


Figure 7.2 The chemical [hypoxic and metabolic stress] and mechanical [hemodynamic forces of shear stress and mechanical stretch] signals and molecular mediators that stimulate angiogenesis.

This thesis investigated transcription factors HIF-1 α and co-factor PGC-1 α which are activated in hypoxia and metabolic signalling pathways. Although HIF-1 α mRNA increased following BFR exercise the response did not differ to the control condition, which has been observed before (Gustafsson *et al.*, 1999; Ameln *et al.*, 2005; Drummond *et al.*, 2008; Fry *et al.*, 2010) suggesting regulation by HIF-1 α does not account for the augmented VEGF expression in response to BFR resistance exercise. However, this hypothesis is limited by being solely based on transcriptional measures of HIF-1 α , which is largely regulated through protein stabilization and nuclear translocation.

Transcription co-factor PGC-1 α regulates VEGF in a HIF-1 α independent manner by downstream co-activation of ERR- α (Arany *et al.*, 2008). This thesis is the first to demonstrate that BFR potentiates the transcript expression of PGC-1 α in response to low load bilateral knee extension exercise (Chapter 5). This finding suggests PGC-1 α regulates the transcription of VEGF with BFR exercise, explaining the absent PGC-1 α and VEGF response to CON exercise. In agreement, mice lacking PGC-1 α in their skeletal muscle failed to increase VEGF expression following acute exercise (Leick *et al.*, 2009), which abolished changes in capillary density in response to exercise training (Chinsomboon *et al.*, 2009).

The 6-fold increase in PGC-1 α mRNA may reflect the enhanced muscle fibre recruitment (Krustrup *et al.*, 2009) and intramuscular metabolic stress caused by BFR during low intensity/load exercise (i.e. PCr depletion, Pi splitting, pH) (Suga *et al.*, 2010). The magnitude of this PGC-1 α mRNA response compares favourably with others in spite of the protocols short duration (~5 min), challenging the notion that prolonged contractile activity is required (>1 h, Russell *et al.*, 2005). The signals operating upstream of PGC-1 α are unconfirmed but could involve activation of AMPK (Olesen *et al.*, 2010) and should be investigated in relation to BFR resistance exercise.

This thesis noted large between subject variability in the HIF-1 α and PGC-1 α mRNA response after BFR exercise. Two individuals demonstrated a robust change in the transcription of HIF-1 α , which has been observed previously in response to similar BFR exercise (Larkin *et al.*, 2012), while one individual, in contrast to the remaining 5 participants, showed no up-regulation in PGC-1 α following BFR resistance exercise. The variation in the level of BFR, which was observed across subjects in chapter 6 (thigh cuff 110 mmHg induces 38-78% BFR) could have influenced muscle oxygen levels (intracellular PO₂) and metabolic stress during exercise (Takarada *et al.*, 2000b; Karabulut *et al.*, 2011) and therefore HIF-1 α protein stabilization and PGC-1 α activation via AMPK. However, it appears the transcription of HIF-1 α is regulated by stimuli independent of tissue hypoxia as the robust change in HIF-1 α mRNA in the two individuals occurred at 4 h post exercise when hypoxia is unlikely (Lundby *et al.*, 2006).

The pro-angiogenic actions of shear stress (eNOS) and mechanical stretch of the tissue (MMP-9) are thought to facilitate angiogenesis (Adair & Montani, 2010). In their absence important processes of capillary growth (EC proliferation and matrix remodelling) are restricted (Haas *et al.*, 2000; Milkiewicz *et al.*, 2005). The transcript expression of eNOS was found to increase after BFR exercise but not CON (chapter 5). Peak eNOS mRNA expression occurred prior to that of VEGF and VEGFR-2, consistent with the hypothesis that shear stress induced NO release functions as an upstream regulator of the VEGF signalling pathway (Milkiewicz *et al.*, 2005). The enhanced eNOS expression may be a consequence of the shear stimulus elicited by reactive hyperaemia following cuff deflation (Gundermann *et al.*, 2012). However, it is also likely due to increased capillary pressure and wall tension (circumferential strain) as a result of raised distal venous pressure with proximal cuff inflation (venous occlusion). Furthermore, since genes are sampled from a muscle homogenate, the expression of eNOS by skeletal muscle rather than EC cannot be excluded. The calcium influx in muscle contraction may also activate both constitutively expressed isoforms of NOS (eNOS and nNOS) (Roberts *et al.*, 1999). Indeed, Larkin *et al.* (2012) reported an increase in nNOS mRNA expression following a similar bout of low load knee extension exercise with BFR. Whilst it cannot be confirmed if enhanced eNOS mRNA is due to shear stress or alternative stimuli, it does suggest the activation of NO pathways that initiate EC proliferation and facilitate angiogenesis by longitudinal splitting (Williams *et al.*, 2006a).

There was no change in MMP-9 mRNA following either bout of low load knee extension exercise (BFR & CON). The indifference between conditions is consistent with the workload-matched protocol eliciting a similar mechanical response in BFR and CON trials and suggests a negligible role of metabolic stress in exercise induced MMP-9 expression (Rullman *et al.*, 2009). The finding that MMP-9 mRNA expression was unchanged may reflect the protocols short duration (5-8 min), which reduced time under skeletal muscle tension (Loenneke *et al.*, 2012) and therefore minimised dynamic stretch of the tissue. This is likely to have inhibited sprouting angiogenesis which requires the remodelling of the extracellular matrix (Stetler-Stevenson, 1999; Haas *et al.*, 2000; Williams *et al.*, 2006b), although without zymographic analysis changes in protease activity cannot be excluded (Rullman *et al.*, 2009). Capillary growth via longitudinal splitting, a process requiring reduced matrix remodelling and

independent from MMP involvement, is more likely to account for the observed increase in K_f in response to BFR resistance exercise (Chapter 4).

7.3.4 Summary

The vascular adaptations to BFR resistance training are likely due to the cumulative molecular effects of each exercise session (Figure 7.3). This involves an initial increase in mRNA transcription in the resistance and capillary vessel endothelium under acute bouts of exercise, which is then followed by structural adaptations (e.g. protein expression, vascular remodelling) induced over periods of sustained training (Jasperse & Laughlin, 2006; Whyte & Laughlin, 2010).

Exposing the conduit artery to short term increases in oscillatory shear and cyclic strain during BFR resistance exercise may activate the ECs, increasing eNOS expression and stimulating outward remodelling through NO-mediated pathways. The enhanced expression of cell adhesion molecules and growth factors, associated with oscillatory shear and ischemic exercise conditions, may facilitate this remodelling process. Further downstream in the resistance vessels exposure to additional hypoxic and/or metabolic signals may activate (via HIF-2 α and/or PGC-1 α) VEGF signalling pathways and play an important role in structural adaptations. This could be due to VEGF modulating eNOS activation with repeated bouts of BFR resistance exercise. In the capillaries the up-regulation of PGC-1 α , eNOS and nNOS (Larkin *et al.*, 2012) may initiate transcription of VEGF and VEGFR-2 and subsequent angiogenesis.

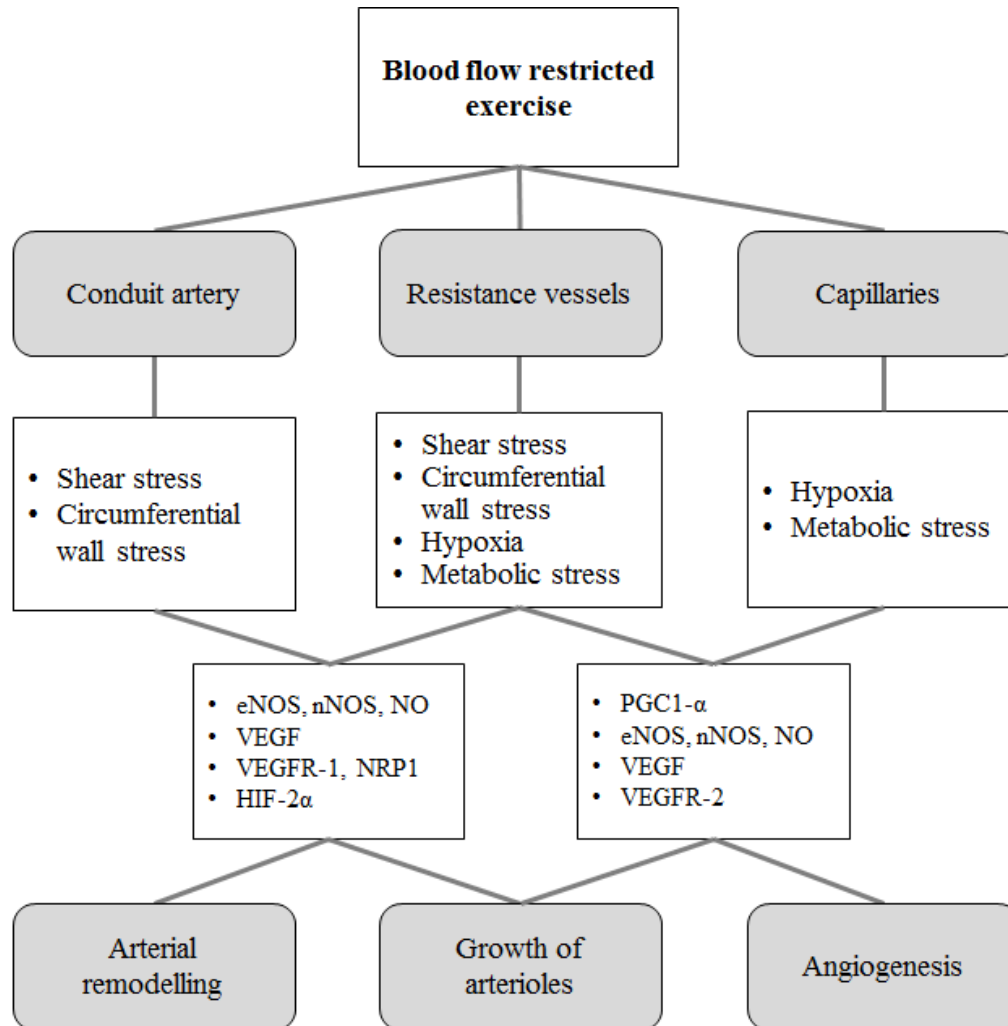


Figure 7.3 The hypothesised physiological signals and molecular mediators of structural adaptations throughout the vascular tree in response to low load resistance exercise with blood flow restriction.

7.4 Functional impact and consideration of vascular adaptations

Peripheral vascular adaptations to exercise training can increase maximal muscle blood flow and gas/nutrient transport. Unfortunately, with no concurrent measures of exercise hyperaemia or endurance performance the functional significance of the vascular adaptations observed in this thesis are unknown. However, there is strong evidence to suggest the enlargement of pre-existing vessels (arterial remodelling) increases blood flow capacity to the downstream

vasculature, while the expansion of the capillary network (angiogenesis) enhances blood-tissue exchange properties. Exercise hyperaemia is determined by the co-ordinated dilation of resistance vessels, feed and conduit arteries (Korthuis, 2011). As such, increased FMD, DC and reactive hyperaemia are associated with enhanced blood flow during isolated-limb exercise (Walther *et al.*, 2008). These adaptations are observed within this thesis and may translate to improvements in aerobic capacity. This is supported by the observation that structural enlargement of the femoral artery and lower leg resistance vessels are positively associated with changes in one-legged $\text{VO}_{2\text{peak}}$ (Miyachi *et al.*, 2001) and whole body $\text{VO}_{2\text{max}}$ (Snell *et al.*, 1987) respectively. Although this thesis noted large changes in peak blood flow, indicative of resistance vessel remodelling, the structural enlargement of the conduit arteries (brachial and popliteal) was modest, limiting the potential increase in blood flow delivery. However, improvements in aerobic capacity are still likely given the increase in K_f (an index of capillarity) which enhances muscle diffusive capacity and contributes more to improved $\text{VO}_{2\text{max}}$ than increases in blood flow delivery (Roca *et al.*, 1992). Furthermore there is evidence that capillarisation improves endurance performance independent of influences on $\text{VO}_{2\text{max}}$, due to the enhanced removal of metabolic by-product during exercise (Coyle *et al.*, 1988). Substantial gains in muscle endurance capacity are associated with enhanced muscle oxygen delivery, following BFR resistance training (Kacin & Strazar, 2011) which was attributed to increased total volume of muscle microvasculature (angiogenesis) or an enhanced metabolic regulation of ascending vasodilatation in the muscle.

This thesis investigated a healthy young population so is limited in its discussion of the therapeutic benefit of these vascular adaptations in more clinical populations. However, improvements in endothelial function, blood flow capacity and blood-tissue exchange/capillarisation are known to reduce the risk of developing cardiovascular disease and metabolic disorders. Such alterations in the vasculature are also associated with improved functional capacity (exercise tolerance) in clinical populations (Robbins *et al.*, 2011).

Whilst the potential benefits are clear, the studies in this thesis and others note a range of individual differences in the acute and chronic response to BFR training. The non-uniform level of blood flow restriction elicited by proximal cuff inflation may be a contributing factor

for variation in angiogenic transcript (mRNA) expression following acute BFR exercise (chapter 5) and arterial remodelling of the resistance (peak reactive hyperaemic blood flow, chapter 4) vessels with BFR training. The cuff pressures utilised in this thesis for upper (80 mmHg) and lower body (110 mmHg) exercise interventions induced a wide range of BFR in male brachial (1 – 68%, relative to baseline) and popliteal arteries (38-78%, relative to baseline). This variation in BFR influences hemodynamic signals, oxygen and nutrient delivery, and the accumulation and clearance rate of local metabolic by-products during exercise (Takarada *et al.*, 2000b; Karabulut *et al.*, 2011). This would have a justifiable impact on training outcomes as conduit and resistance vessel adaptation is related to the magnitude and pattern of shear stress and capillary growth is proportional to metabolic activity. Ensuring an equal and appropriate level of BFR between participants is therefore important to achieve consistent gains from exercise training. Participants with higher blood pressure (MAP, DBP) and BMI require higher cuff pressures to elicit a partial level of BFR (~60%). The usefulness of basing the pressure on the individuals' limb circumference is questionable since the variation in limb size that exists between participants in an asymptomatic population (as observed in chapter 6) is small, and has limited impact on eliciting a partial level of BFR. Despite measuring recognised predictor variables for complete arterial occlusion pressure, the majority of the variance in partial BFR pressure was unexplained in chapter 6. More may depend on local vascular function at the site of cuff inflation.

It is interesting to note that the BFR stimulus utilised in this thesis during upper (80mmHg) and lower (110mmHg) body exercise interventions did not elicit conduit artery constriction. Higher occlusion pressures may induce vasoconstriction due to low-flow, which is a response mediated by endothelin-1 (Spieker *et al.*, 2003) and/or through inhibiting EDHF and prostaglandins (Gori *et al.*, 2008). BFR training studies should be wary of applying higher external cuff pressures, which induce artery constriction, and with repeated use, cause potential endothelial dysfunction.

7.5 Limitations

The limitations of this thesis are acknowledged. With the inclusion of healthy habitually active male and female volunteers the findings cannot be extrapolated to more sedentary or clinical populations. Only the relevant application of this exercise mode in training environments is comment upon, while further evidence is required for its potential use as a therapeutic treatment.

Although the dilatory response to ischemic exercise is a valid indicator of conduit artery structure (Naylor *et al.*, 2005), endothelium independent testing via sublingual glyceryl trinitrate may have been more conclusive. A limitation of venous occlusion strain-gauge plethysmography is that it does not allow us to elucidate the exact mechanisms behind changes within the vasculature. Intra-arterial infusion of endothelium dependent agonists (acetylcholine) and NOS antagonists (L-NMMA) would enable examination of resistance vessel endothelial function (Wilkinson & Webb, 2001). However the necessity for arterial cannulation prevented the use of this methodology.

Measuring changes in blood circulatory factors during BFR exercise (chapter 5) and training (chapter 3 & 4) may have allowed the quantification of relevant chemical physiological stimuli and provided a mechanistic insight behind vascular adaptations. Early attempts were made to measure plasma nitrate/nitrite by chemiluminescence (using Sievers nitric oxide analyser; NOATM 280i) as an index of NO production and eNOS activity. However, due to the complexity of NO metabolism, its rapid half-life and various contaminants from non-vascular sources, the assessment of endothelial NO production proved to be unsuccessful. Direct sampling of vascular EC from the brachial artery was considered but was ultimately beyond the scope of this thesis. This would have enabled the determination of EC protein expression by quantitative immunofluorescence, and provided novel insight into the molecular mechanisms of vascular adaptations. Sampling the surrounding muscle tissue is a valid alternative, because the musculature is highly vascularized and it represents the primary tissue affected by exercise (Lammers *et al.*, 2012). Some of our responsive genes are localized on the EC (VEGFR-2, eNOS) demonstrating targeted activation. Previous investigations that have measured the same transcript expression (VEGF, VEGFR-2 & eNOS mRNA) and report a

similar response to acute exercise, observe capillarisation (Hellsten *et al.*, 2008; Høier *et al.*, 2010; Hoier *et al.*, 2012) and arterial remodelling (Lammers *et al.*, 2012) with exercise training. This supports the assumption that changes in muscle gene expression may lead to processes of structural vascular adaptation.

This thesis has observed upper and lower body vascular adaptations to localised exercise involving different muscle groups with the utilisation of different occlusion protocols. BFR cuff pressure was based on the current literature and pilot observations, and designed to elicit venous occlusion and reduce arterial inflow during exercise without inducing overt premature fatigue (Fahs *et al.*, 2012b). However, it appears there was over compensation for the disassociation between tourniquet pressures, cuff width, underlying soft-tissue pressure and limb circumference (Shaw & Murray, 1982) since chapter 6 confirmed the dissociation between the BFR stimulus in upper and lower limbs. On average, males experienced 28% BFR in the brachial compared to 60% BFR in the popliteal at cuff pressures of 80 mmHg and 110 mmHg (chapter 6).

The move from an upper to lower limb exercise model was made for consistency with previous BFR investigations and because of the opportunity to observe the adaptation at different levels of the vascular tree. However, we failed to perform repeated measures of calf K_f , which would have identified the time course of changes in capillarity in relation to conduit and resistance vessel adaptation.

Finally, this thesis implemented two forms of control condition, where exercise was matched (load and repetitions) but without BFR (chapters 3 & 5) or no exercise was performed (chapters 4). Although this reflects inconsistencies in research design it allowed the evaluation of the additional benefit of BFR to low load resistance exercise alone (chapter 3 & 5) and the potential cross-transfer effect elicited by this mode of exercise (chapter 4). In each controlled condition the acute response (chapter 5) and chronic adaptation (chapter 3 & 4) of the vasculature was absent. The observations from chapters 3 and 5 signified the inability for low load resistance exercise alone to stimulate vascular adaptations, in line with traditional concept that an intensity and/or volume threshold exists (Goto *et al.*, 2003). Alternatively, the study design utilised in chapter 4 allowed the comparison of responses between the BFR trained and

untrained limb to assess evidence of a cross-transfer effect. Systemic vascular adaptations may have occurred in response to localised BFR exercise due to its association with increased circulatory VEGF and progenitor cell release (Takano *et al.*, 2005; Sandri *et al.*, 2011; Larkin *et al.*, 2012) and observations of systemic endothelial protection with remote ischemic preconditioning (Bailey *et al.*, 2012). Despite this we did not observe any vascular changes in the non-trained leg. This suggests regional rather than systemic factors mediate vascular adaptations or alternatively circulatory factors are drawn only to the site of EC activation (Zampetaki *et al.*, 2008). Exercise involving larger muscle groups may be required to achieve the threshold of hemodynamic and circulatory factors that would be associated with a systemic vascular adaptation (Padilla *et al.*, 2011). Furthermore, it is likely that the promotion of capillarisation via remote angiogenic factors (VEGF, EPC) was reduced given the absent metabolic demand and ischemia in the non-exercised control limb. Studies implementing a load matched non-BFR exercise control may find results differ.

7.6 Directions for future research

The findings of this thesis have highlighted the potential benefit of low load resistance exercise with BFR on the peripheral vasculature. The observations made have generated new questions that future research should aim to address.

Defining the shear stimuli: It is vital that future studies characterise the blood flow and shear rate patterns during BFR resistance exercise. This requires measurement of both antegrade and retrograde components to quantify average flow/shear and oscillatory index. This describes the exposed conditions on the ECs and subsequently the stimuli for adaptations in vascular function.

Acute and chronic vascular adaptations to BFR resistance exercise: Further studies are needed to examine the impact of changes in shear patterns during a BFR exercise bout on post exercise measurements in vascular function. Prolonged exposure to disturbed flow (20 min) can acutely decrease vascular function (increase vasoconstriction and decreased vasodilation) (Rakobowchuk *et al.*, 2013) but the effect of short duration (>10 min) BFR resistance exercise

is unknown. Multiple measurements of FMD post exercise would determine whether a biphasic response exists in accordance with current hypotheses (Dawson et al 2013). These acute responses could be observed alongside chronic effects of training to determine changes in sensitivity to BFR exercise stimulus. Longitudinal investigations should monitor the vasculature over longer duration BFR training interventions. The functional significance of vascular adaptations should be determined by performing assessments of blood flow during exercise and endurance performance. Finally, more than one muscle group should be targeted by exercise training to reflect a real-world setting and establish systemic vascular adaptations.

Cell-based methodologies: Future studies should incorporate cell-based methods (Endothelial biopsy, EPCs) for a comprehensive evaluation of endothelial cell biology in response to BFR exercise conditions. Endothelial biopsy is a minimally invasive technique whereby ECs are harvested (using a J shaped wire) through a cannula in a superficial vein or artery (Fadini & Avogaro, 2010). Measurement of protein expression can be performed using quantitative immunofluorescence analysis (Colombo *et al.*, 2002). Protein markers of NO production (eNOS), oxidant enzymes (NADPH, xanthine oxidase), antioxidant defences (catalase, superoxide dismutase), oxidative stress (nitrotyrosine) and inflammation (cyclooxygenase-2, nuclear factor κ B) could explain changes in endothelial function (NO bioavailability) and establish the mediators of vascular adaptations with BFR resistance exercise. Although this technique has been utilised in cross-sectional investigations (e.g. (Silver *et al.*, 2007) its application to evaluate training interventions is novel.

Repair of the endothelium and formation of new blood vessels is promoted by the release of bone-marrow derived EPCs. Exercise triggers the mobilisation of EPCs from bone marrow, increasing the number in circulation. This occurs primarily in response to elevated VEGF with a minor contribution from shear stress induced NO liberation. Exercise-induced VEGF expression is enhanced under BFR conditions (chapter 5) which may augment peripheral EPC numbers. This suggests BFR exercise may enhance vascular regenerative capacity, offering a therapeutic advantage over other exercise modes. Combined evaluation of endothelial function (FMD), regenerative capacity (EPC) and biochemical pathways (biopsy) would provide a comprehensive study of the endothelium in response to BFR exercise conditions.

Isolating physiological stimuli: Future studies should attempt to isolate the physiological stimuli (chemical vs. hemodynamic) during exercise and determine regional vascular adaptations. This would establish if compensatory vascular mechanisms occur in the absence of principle physiological signals. Investigations could manipulate the methodology adopted in chapter 5, whereby establishing the acute angiogenic response to multiple sets of BFR low load resistance exercise when pressure is applied constantly throughout rest periods (continuous - as employed in this thesis) or released at the completion of each exercise set (discontinuous). If multiple sets are matched for work done (the same repetitions performed between protocols), continuous occlusion will induce greater local ischemia, while discontinuous occlusion higher shear stress. This design will assess if chemical or hemodynamic mechanical factors contribute more to exercise induced angiogenesis.

Adaptation in females: Studies investigating BFR exercise and vascular adaptations to training have almost exclusively relied on male participants. Gender differences in endothelial function and vascular reactivity exist due to sex hormones (Sader & Celermajer, 2002), yet it is not known if proportional exercise-induced vascular adaptations occur. Hormonal status can be measured in female participants to ensure experimental trials are conducted during the midfollicular phase of their menstrual cycle to minimize the potential effects of monthly estrogenic variation on study outcomes.

Therapeutic application: BFR exercise appears to be a viable means of improving strength in injured (Ohta *et al.*, 2003), older (Takarada *et al.*, 2000b; Yokokawa *et al.*, 2008; Karabulut *et al.*, 2010) and clinical (Gualano *et al.*, 2010; Satoh, 2011) populations who show intolerance to high mechanical loading. Previous reports suggest older people experience an increase in reactive hyperaemia (Patterson & Ferguson, 2011) and arterial compliance (Ozaki *et al.*, 2011) following BFR training. Further investigation into vascular adaptations and effect on muscle blood flow capacity are warranted. Improvements in the latter reduce atheroma formation and preserve skeletal muscle health, reducing the risk of developing cardiovascular disease and metabolic syndromes. The time course or magnitude of adaptation may differ in healthy subjects versus clinical populations.

7.7 Conclusions

The studies contained in this thesis demonstrate potential functional and structural changes in conduit arteries, resistance vessels and the capillaries in response to low load resistance exercise with BFR. The time courses of these adaptations match those seen with higher intensity exercise without BFR, while the magnitude of change compares favourably in the resistance and capillary vessels. This finding supports the theory that chemical signals activated by local tissue hypoxia and/or metabolic activity mediate adaptation of the downstream vasculature. For the first time it has shown that low load resistance exercise with BFR can up-regulate PGC-1 α and eNOS mRNA, which may initiate transcription of VEGF and VEGFR-2 and subsequent angiogenesis. The hemodynamic signals during low load resistance exercise with BFR initiate conduit artery remodelling but require further classification. Future research should combine measures of endothelial function (FMD), regenerative capacity (EPC) and biochemical pathways (biopsy) for a comprehensive study of the endothelium in response to BFR exercise conditions. The functional significance of the vascular adaptations on endurance performance following BFR exercise training should be established.

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Appendix A: Participant Information

Regional vascular adaptations to low load resistance training with blood flow restriction

Name, address, email and contact number of Main Investigator

Dr Richard Ferguson	R.Ferguson@lboro.ac.uk	01509 226333
Loughborough University		
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Name, address, email and contact number of all other investigators/supervisors

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Study Overview

The aim of the study is to examine the effect of short-term (6 week) plantar flexion low load resistance training with blood flow restriction, on calf muscle strength and regional vascular adaptations. The investigation will also compare responses between the trained and untrained limb.

Who is doing this research?

Dr Richard Ferguson is the senior investigator leading the project. Julie Hunt is a PhD student who will be responsible for the day-to-day running of the experiment.

Are there any exclusion criteria?

You will be one of 12 male participants (18-35 years). Prior to any experiments you will complete a health and physical activity questionnaire to ensure you are able to take part. You

must be physically active, i.e. perform three or more 30 minute exercise sessions per week. You must have no history of cardiovascular, metabolic or haematological disorders.

Once I take part, can I change my mind?

It should be noted that you will be free to withdraw from the study at any time without giving a reason. If you are a student of Loughborough University your decision to participate / not participate / withdraw will not have any bearing on your academic progress.

Will I be required to attend any sessions and where will these be?

You will be asked to come to the laboratory on 24 separate occasions; once for a familiarisation session, four times for the main experimental tests and 18 times for training. The location is the Cardiovascular Laboratory (room HE2.42) in the Clyde Williams building (location no. 105 on the University Campus map) on the Loughborough University campus.

How long will it take?

You will attend 1 familiarisation session (1 hour) and 4 experimental testing sessions (2-4 hours). You are required to train 3 times per week for 6-weeks (18 training sessions) each session lasting approximately 10-min. The total time will be ~16 hours over an 8 week period.

Is there anything I need to do before the sessions?

It is important that conditions are kept similar for all experimental testing sessions and for this reason you should maintain a similar pattern of physical activity and normal diet habits during the study period. All main testing sessions will be conducted in the morning (~7-11am) after a 10 hour overnight fast. You are asked to refrain from vitamin supplementation for 72 hours, and no exercise, caffeine, alcohol or tobacco 12 hours, prior to vascular tests.

What type of clothing should I wear?

On each visit you should bring comfortable clothing suitable for exercising in. Shorts are required for ultrasound imaging behind the knee. There will be a private changing room made available for you.

What will I be asked to do?

Experimental Procedures

During your familiarisation visit you will be given a demonstration of the testing procedures. You will be instructed on the proper use of the resistance exercise equipment and perform a practice blood flow restricted training session. Measures of your calf strength and regional vasculature will be conducted during experimental testing sessions according to procedures outlined below.

Vascular measures

Popliteal artery function and structure will be assessed through non-invasive techniques using ultrasound. You will lie on your front with your knee fully extended. A blood pressure cuff will be placed around your calf. The ultrasound probe will be positioned over the artery (behind your knee) and held stable.

Flow mediated dilation (FMD): For the first measurement the cuff around the calf will be inflated to a high pressure (200mmHg) for 5 min. Ultrasound recordings will be performed 30 s before cuff deflation and continue for 5 min thereafter. You will then be given a 15-min rest period.

Dilatory capacity (DC): For the second measurement the cuff will be placed above your knee and inflated to a high pressure (200mmHg) for 5 min. You will perform ankle extension exercise during the middle 3 min. Ultrasound recordings will be measured 30 s prior to cuff deflation and will continue for 5 min thereafter. The procedure will be repeated on both legs.

Capillary filtration: Calf filtration capacity, resting blood flow and venous pressure will be assessed using a non-invasive technique called strain gauge plethysmography. You will lie in the supine position (lying flat on your back) with your leg supported in line with your heart. A blood pressure cuff will be placed above your knee. You will rest in this position for a period of 10 minutes. A strain gauge will be positioned around the widest part of your calf. Resting blood flow measurements will be taken by inflating the cuff to a low pressure (50 mmHg) for 7-8 s, followed by a deflation period of 45 s. This process will be repeated three times.

Remaining in the supine position calf filtration capacity will be assessed. The blood pressure cuff will be inflated in cumulative pressure steps of ~8mmHg and maintained for 4-min. In total 8 incremental steps will be completed resulting in a total period of 32 minutes. The cuff will then be immediately deflated and the strain gauge and blood pressure cuff removed.

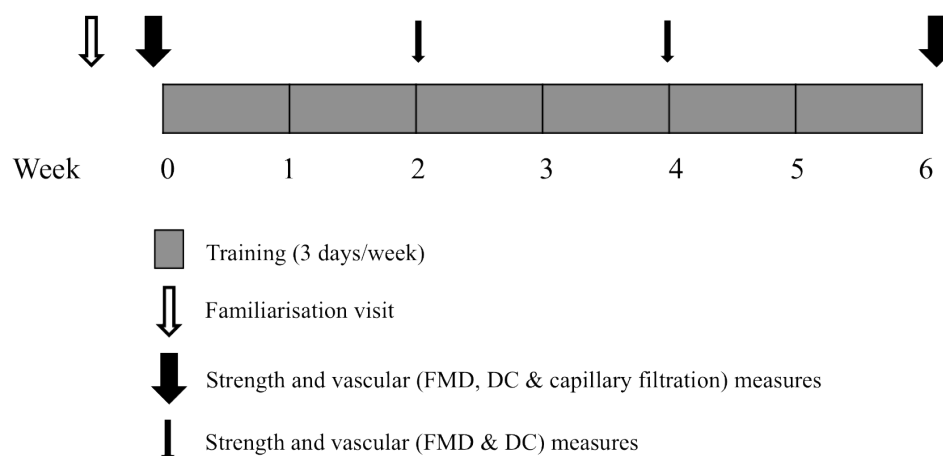
Calf strength

The maximum weight you can lift during a calf raise exercise (plantar flexion) will be determined on a leg press machine. You will lie on your back and attempt to lift the weight rack by performing a calf raise. Lifts will begin at 60-80% of your perceived maximum and the load gradually increased until failure. A four-minute recovery period between attempts will be allowed. The highest successful lift is defined as your one repetition maximum (1RM).

Exercise training

Training will be performed 3 times per week for a total of 6 weeks. Each training session will involve 3 sets of unilateral calf raise exercise, at a frequency of 20 contractions per minute (1.5 s lifting 1.5 s lowering) performed to volitional fatigue. Each set will be separated by 1-min inactive recovery. During exercise a pressure cuff will be placed above the knee and inflated to a moderate pressure (110mmHg). This is promptly released after the exercise is completed. Blood flow will be restricted for ~3-8 minutes in total.

Study timeline



Possible benefits of taking part

Free strength training and information on vascular health. You will hopefully find the experience of being a participant for a research study extremely interesting. You will also be playing your own part in contributing to the existing body of scientific knowledge.

Are there any risks in participating?

Resistance training, especially with blood flow restriction, will result in local muscle fatigue. The blood flow restriction may also cause slight discomfort but this sensation disappears immediately on release of the cuff. Side effects that have been reported during resistance training with blood flow restriction include bruising and temporary numbness. Other extremely rare side effects have included venous thrombosis (a blood clot that forms within a vein), rhabdomyolysis (skeletal muscle damage) and pulmonary embolism (blockage of the main artery of the lung).

All physiological measurements are non-invasive. The vascular measurements require very high cuff inflation pressure and the performance of muscle contractions during total blood flow restriction. This will result in severe local muscle fatigue. All responses are transient and the investigators involved are vigilant in ensuring the participants comfort and safety at all times.

Will my taking part in this study be kept confidential?

All data will be dealt with under the strictest of guidelines and according to the Data Protection Act. Participants will be numerically coded and data will be discussed only amongst the lead investigators. The results of the study will be used to formulate relevant conclusions. After completion it may be used in further publications, however confidentiality will be preserved.

What if I am not happy with how the research was conducted?

The University has a policy relating to Research Misconduct and Whistle Blowing which is available online at [http://www.lboro.ac.uk/admin/committees/ethical/Whistleblowing\(2\).htm](http://www.lboro.ac.uk/admin/committees/ethical/Whistleblowing(2).htm)

Appendix B: Informed Consent Form

(to be completed after Participant Information Sheet has been read)

Time course of regional vascular adaptations to low load resistance training with blood flow restriction

- The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethical Advisory Committee.
- I understand that a duplicate copy of this form will be kept by the University.
- I have read and understood the information sheet and this consent form.
- I have had an opportunity to ask questions about my participation.
- I understand that I am under no obligation to take part in the study.
- I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.
- I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others.
- I agree to participate in this study.

Your name _____

Your signature _____

Signature of investigator _____

Date _____

Appendix C: Health Screen Questionnaire

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research.

Please complete this brief questionnaire to confirm your fitness to participate:

1. At present, do you have any health problem for which you are:

(a) on medication, prescribed or otherwise	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(b) attending your general practitioner	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(c) on a hospital waiting list	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>

2. In the past two years, have you had any illness which required you to:

(a) consult your GP	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(b) attend a hospital outpatient department.....	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(c) be admitted to hospital	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>

3. Have you ever had any of the following:

(a) Convulsions/epilepsy	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(b) Asthma	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(c) Eczema	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(d) Diabetes	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(e) A blood disorder	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(f) Head injury	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(g) Digestive problems	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(h) Heart problems	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(i) Problems with bones or joints	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(j) Disturbance of balance/coordination	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>

(k) Numbness in hands or feet	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(l) Disturbance of vision	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(m) Ear / hearing problems	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(n) Thyroid problems	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(o) Kidney or liver problems	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(p) Allergy to nuts	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>

4. **Has any**, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise? Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

.....

.....

5. Allergy Information

(a) are you allergic to any food products?	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(b) are you allergic to any medicines?	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>
(c) are you allergic to plasters?	Yes	<input type="checkbox"/>	No	<input type="checkbox"/>

If YES to any of the above, please provide additional information on the allergy

.....

.....

6. Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name:.....

Telephone Number:.....

Work ☐ Home ☐ Mobile ☐

Relationship to Participant:

7. Are you currently involved in any other research studies at the University or elsewhere?

Yes ☐ No ☐

If yes, please provide details of the study

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