The acute effects of shear rate manipulation on vascular function in a healthy population

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Abstract

Disease of the cardiovascular system is responsible for the majority of worldwide deaths. The progression of atherosclerosis is pertinent to increased risk of an acute coronary event. An understanding of all factors that contribute to the formation of atherosclerotic plaque is essential for future primary and secondary prevention of cardiovascular diseases.

It is now well established that shear rate (SR), the quotient of blood flow velocity and vessel diameter, is responsible for both positive and negative adaptation of the vasculature. Previous data has identified that inflation of a cuff around the forearm induces an increase in retrograde SR, which is detrimental to vascular function.

Despite previous data highlighting the acute negative effects of distal cuff inflation on vascular function, little is known about the effects of proximal cuff inflation. Data from study 1 of this thesis indicates that proximal cuff inflation alters SR pattern to a similar degree as distal cuff inflation. However, vascular function, as measured by the established technique of flow mediated dilation (FMD) and the novel technique of low-flow mediated constriction, was not significantly attenuated in the proximal condition. This contrasts with the impacts of distal cuff inflation, which mirrored previous findings.

The results from study 1 informed study 2, where a venous occlusion plethysmography (VOP) protocol was performed. Venous occlusion plethysmography has been widely used in research and clinical settings to assess blood flow in the periphery. The universal assumption that arterial inflow is not altered during VOP was questioned. The findings suggest that arterial inflow is, in fact, reduced during VOP. Moreover, it was found that retrograde SR was significantly increased. However, this did not impact upon vascular function as measured by FMD.

In summary, despite previous evidence that prolonged and persistent increase in retrograde SR is detrimental to vascular function, neither proximal cuff inflation, nor VOP protocols, negatively affected arterial function. These novel findings highlight the complexity of haemodynamic physiology and suggest that factors other than retrograde SR may influence acute vascular function.

Acknowledgments

I began writing my acknowledgments in a traditional and formal style, but decided it would be better to write something more heartfelt that I could reflect on in years to come.

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And to Andrew, my rock… here's to the future!

Declaration

I declare that the work contained in this thesis is entirely my own. Some of the work has been published in peer-reviewed journal. Beyond assistance with data acquisition and input from my supervisors no other individual has examined the findings presented herein.

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List of Abbreviations

BP	Blood pressure
Ca^{2+}	Calcium
cGMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
EDHF	Endothelium derived hyperpolarising factor
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FMD	Flow mediated dilation
GTN	Glyceryl trinitrate
GTP	Guanosine triphosphate
LMM	Linear mixed model
L-NMMA	NG-monomethyl-L-aginine
NO	Nitric oxide
HR	Heart rate
ROI	Region of interest
sGC	Soluble guanylate cyclase
SR	Shear rate
SNP	Sodium nitroprusside
SBP	Systolic blood pressure
+ve	Positive
-ve	Negative

CHAPTER 1 - Background

1.1 Background

Cardiovascular disease (CVD) is the world's leading cause of mortality, attributing to ~30% of all deaths (Alwan, 2011). The umbrella term of CVD encompasses a range of conditions that adversely affect the circulation, including coronary heart disease, cerebrovascular disease and peripheral arterial disease. Risk factors for cardiovascular disease include age, family history, smoking status, hypertension, hyperlipidaemia and obesity, with more recent novel risk factors including type 2 diabetes and physical inactivity (Lee *et al.*, 2012). The underlying link between these cardiovascular risk factors is the process of atherosclerosis, which leads to the development of CVD. Atherosclerosis describes the formation of lesions in the coronary and peripheral conduit arteries (Libby and Theroux, 2005). Although the progression of lesion formation is complex and multi-faceted, it is initially stimulated by an inflammatory response to acute damage of the inner lining of the artery, the endothelium (Libby, 2002). The progression of atherosclerosis specifically, is pertinent to increased risk of acute coronary events.

The earliest detectable manifestation of atherosclerosis is endothelial dysfunction, which occurs long before the clinical presentation of CVD (Quyyumi, 2003). Once thought of as merely a semi-permeable membrane (Versari *et al.*, 2009), the mono-layered endothelium is crucial for maintaining vascular tone (Furchgott and Zawadzki, 1980) and arterial health, principally through the production of anti-atherogenic autacoids (Moncada *et al.*, 1991; Cooke and Tsao, 1994). Endothelial dysfunction is characterised by a vascular phenotype that is predisposed to atherosclerosis (Bonetti *et al.*, 2003) and its assessment is therefore believed to represent a useful 'barometer' of cardiovascular risk (Vita and Keaney, 2002). As a result, several studies have developed and validated techniques that claim to examine endothelial function. A frequently adopted, popular and non-invasive technique to examine

endothelial function relates to the assessment of flow mediated dilation (FMD), a well established prognostic marker of coronary disease risk (Green *et al.*, 2011). More recently, the technique of low-flow mediated constriction (L-FMC) has been identified as a complementary adjunct to FMD, which can be assessed concurrently (Gori *et al.*, 2008). Although in its infancy, it has been suggested that L-FMC used alongside FMD may account for total dilator/constrictor vessel reserve, which may better assess vascular function and aid our understanding of endothelial dysfunction. However, relatively little is known about the L-FMC technique.

An important factor that inherently influences vascular health is blood flow through the artery and its direct impact on the endothelium. Arterial blood typically flows in antegrade direction (forward) throughout the majority of the cardiac cycle, although many healthy peripheral arteries demonstrate high resistance bi/triphasic waveforms with periods of retrograde (backward) flow during diastole (Marinelli *et al.*, 1979). However, periods of increased retrograde flow can be detrimental to the health of the endothelium.

There is a wealth of literature investigating endothelial responses to both acute and chronic increases in retrograde flow at a cellular level. *In vivo* studies, however, are less abundant. Previous *in vivo* studies have used cuff inflation to manipulate blood flow patterns and subsequently shear stress/rate (Thijssen *et al.*, 2009b; Tinken *et al.*, 2009; Schreuder *et al.*, 2014). These studies have served to highlight that acutely, retrograde flow/shear rate is potentially detrimental to vascular function, as measured by FMD using Doppler ultrasound. In these studies, an increase in retrograde shear rate (SR), was induced via inflation of an automatic pneumatic inflation/deflation cuff *below* the area of ultrasound measurement. This manipulation is believed to result in an increase in peripheral resistance, causing an

increased retrograde SR in the upstream artery. However, no previous study has examined the effect of such cuff placement on the SR pattern and vascular function *below* the cuff. Theoretically, since flow/SR is decreased above the cuff, it could be hypothesised that flow/SR may also be decreased *below* an inflated occlusion cuff. However, little is known about the exact stimuli that mediate acute adaptation, although it is likely that shear, pressure and humeral/neural stimulation all contribute (Padilla *et al.*, 2010; Newcomer *et al.*, 2011) . It is unknown how these stimuli differ between distal and proximal cuff placements, and whether this may have clinical implications. By fully understanding what mediates vascular adaptation, interventions that aid vascular augmentation, and ultimately cardiovascular health, can be better designed and implemented.

Although cuff inflation manipulates flow/SR artificially, a real world example of when flow/SR may be altered is in the case of an arterial stenosis. In stenotic areas of a vessel, flow velocities and flow/SR patterns are altered before, at, and after the site of stenosis. In other cardiovascular conditions, including systemic heart, kidney and liver failure as well as localised conditions such as thrombophlebitis and varicose veins, peripheral oedema is common. In these patients, increases in pressure around the blood vessels, increased microvascular resistance and venous pooling may all affect arterial shear rate patterns and subsequently alter arterial function and health. Many patients with cardiovascular disease such as hypertension and diabetes often present with increased arterial wall stiffness (Laurent and Boutouyrie, 2007). Reduced elastic recoil in the arteries alters pulse wave reflections and ultimately shear rate patterns. Risk of atherosclerosis and cardiovascular disease in these patients is greatly increased (Laurent *et al.*, 2006). It is therefore pertinent to fully understand the effects of altering shear patterns on vascular function.

There are a number of different methods used to assess vascular function. Venous occlusion plethysmography (VOP) is a traditional non-invasive measure of vascular function that dates back to the early 20th century and is largely based on repeated inflation of blood pressure cuffs to sub-diastolic levels. Plethysmography is typically used to assess vascular function and the local effects of vasoactive drugs in an *in vivo* model (Wilkinson and Webb, 2001) and is the historical gold standard technique. Venous occlusion is induced by intermittent inflation of a sub-diastolic blood pressure cuff where it is assumed that whilst venous flow is congested, arterial inflow remains unaffected. Furthermore, an additional cuff is inflated to a supra-systolic pressure (~220 mm Hg) around the wrist, to arrest the hand circulation. Interestingly, previous studies indicate that (repeated) inflation of blood pressure cuffs to sub-diastolic levels may interfere with the blood flow to the arm. More specifically, inflation of the blood pressure cuff to sub-diastolic levels increases retrograde shear rate. This shear pattern is typically associated with impairment in acute endothelial function. It is therefore possible that VOP which is used as a measurement of vascular function may, in itself, influence vascular function through alteration of arterial shear rate patterns.

Importantly, many previous studies investigating the effects of cardiovascular drugs on the vasculature have adopted VOP as their primary outcome measure. Therefore, if fundamental assumptions regarding the VOP technique are not upheld, then this could have important clinical implications for findings in thousands of previous papers examining vascular function and health. To date, no study has investigated whether a venous occlusion plethysmography protocol (i.e., repeated inflation of blood pressure cuffs to sub-diastolic levels) in itself alters shear rate pattern and if this in turn affects artery function as assessed by flow mediated dilation.

1.2 Aims and Objectives

The specific aims of this thesis are:

1. To investigate the acute effects of shear manipulation *via* cuff inflation proximal and distal to the brachial artery to a sub-diastolic level on shear pattern within the brachial artery.
2. To investigate the effects of shear rate manipulation *via* cuff inflation proximal and distal to the brachial artery on brachial artery vascular function using the established method of flow mediated dilation and the novel technique of low-flow mediated constriction.
3. To explore whether the traditional venous occlusion plethysmography protocol, involving repeated cuff inflation to sub-diastolic level, alters arterial inflow and whether this protocol affects brachial artery vascular endothelial function.

The above aims will be achieved through the following objectives:

1. Aims 1 and 2 will be addressed by conducting a within-subjects study examining flow mediated dilation and low-flow mediated constriction before and after brachial artery shear manipulation *via* proximal and distal cuff inflation.
2. Aim 3 will be addressed by conducting a pilot study investigating the effects of a venous occlusion plethysmography protocol on shear pattern and flow mediated dilation.

CHAPTER 2 - Review of the literature

2.1 The cardiovascular system

The human cardiovascular system is responsible for serving all living tissue with oxygen rich blood. De-oxygenated blood returned to the heart is pumped through the pulmonary circulation where it is oxygenated and returned to the heart before being distributed around the body.

2.1.1 Anatomy of the cardiovascular system

In essence, the heart serves as a pump, while the blood vessels act as delivery pipes. Electrical stimulation initiated in the sinoatrial node, moves through the atrioventricular (AV) node, bundle of His, right AV branch and Purkinje fibres, synchronising the contraction of firstly the atria and then the ventricles. Relatively de-oxygenated blood is returned to the heart via the venous system and enters the right atrium *via* the superior/inferior vena cava. The right side of the heart propels blood through the right ventricle into the pulmonary circulation where it is oxygenated and returned to the left atrium. Simultaneously, blood is mechanically driven into the left ventricle via contraction of the left atria, where it is then ejected by the left ventricle into the systemic arterial system *via* the ascending/descending aorta.

Nutrient rich blood is supplied to the peripheral vascular beds firstly through large conduit arteries. These large elastic vessels split into smaller muscular conduit arteries and then resistance vessels (or arterioles) before splitting further into capillaries. The microvasculature serves the muscle beds, skin and vital organs with blood before turning into venules and veins which return of blood to the right atria.

2.1.2 The cellular makeup of conduit arteries

Conduit arteries vary significantly in size, from the large common carotid artery (5-7 mm) (Krejza *et al.*, 2006) to the relatively small radial artery (2-3mm) (Saito *et al.*, 1999). These arteries are made up of three distinct layers; tunica adventitia (outer layer), tunica media (middle layer) and tunica intima (innermost layer) (Figure 2.1). The tunica adventitia and media are composed of varying quantities of elastic fibres, connective tissue and smooth muscle fibres. The tunica intima comprises of a single layer of squamous cells called the endothelium, which lie alongside a bed of elastic and connective tissue.

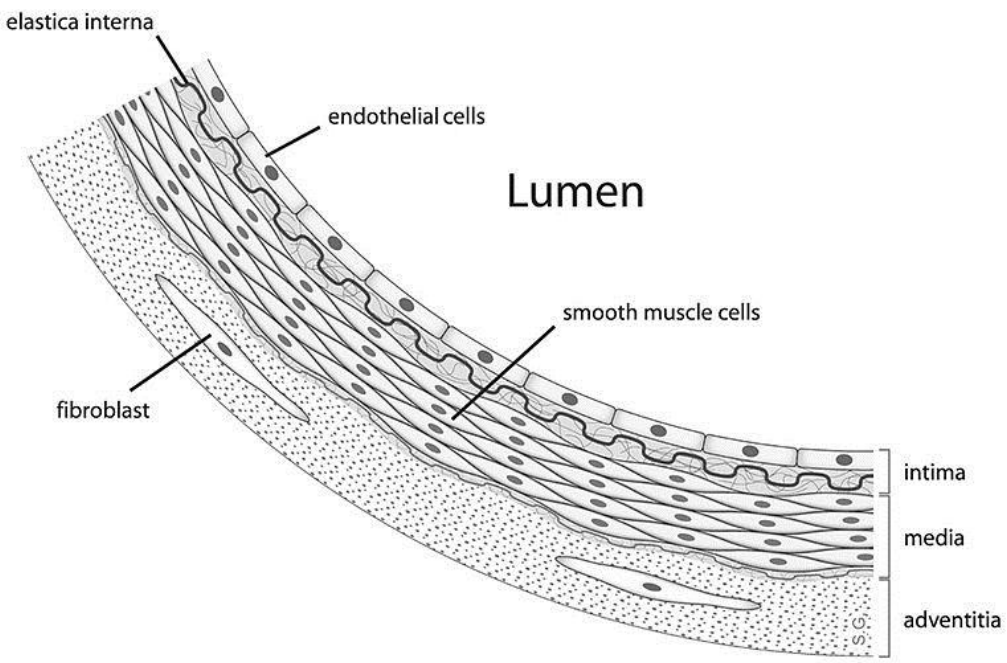

Figure 2.1. The anatomy of an artery
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The mono-layered endothelium coats the inner lining of the entire vascular tree and is the direct interface between haemodynamic flow and the vessel wall (Chiu and Chien, 2011). Due to its location, the endothelium is directly exposed to changes in blood flow and shear stress.

2.1.3 Haemodynamics

The volume of blood that passes through a section of the circulation at any particular time is denoted by the equation:

$$\text{Blood Flow } (ml \cdot min^{-1}) = \text{Mean Blood Velocity } (cm \cdot min^{-1}) \times \text{Vessel Cross Sectional Area } (cm^2)$$

The equation is based upon the assumption that blood flow is laminar and unidirectional and that the artery is perfectly cylindrical. Whilst this remains true in larger conduit arteries (i.e., the common carotid), in the smaller brachial artery, oscillatory flow (with periods of retrograde i.e., backwards, flow during diastole) is normal at rest (Halliwill and Minson, 2010). Blood flow through a vessel is also dependent upon the change in pressure and the downstream resistance.

Although the volume of blood flowing through a particular vessel is important, the stimulus for arterial adaptation is shear stress, the frictional force flowing blood cells elicit against the endothelium. *In vivo* measurement of shear stress (equation below) is difficult as it is dependent on accurate blood viscosity and velocity recordings close to the artery wall (Wootton and Ku, 1999). It is especially challenging to measure viscosity as this would involve invasive techniques and specialist equipment (Parkhurst *et al.*, 2012).

$$\text{Shear Stress (Pa)} = \text{Viscosity } (Pa \cdot s) \times [\text{Mean Blood Velocity } (cm \cdot s^{-1}) / \text{Vessel Diameter (cm)}]$$

In vascular physiology, shear stress is most commonly estimated by shear rate (SR), the quotient of blood flow velocity and vessel diameter, denoted by the equation:

$$\text{Shear Rate } (s^{-1}) = 4 \times \text{Mean Blood Velocity } (cm \cdot s^{-1}) / \text{Vessel Diameter (cm)}$$

Although blood flow and SR are innately related, they only correlate in vessels of the same or similar size (Pyke and Tschakovsky, 2005). In vessels of differing sizes, equal blood flow can result in very different shear rate (Table. 2.1).

Table 2.1. Example of the effect of diameter on shear rate

Variable	Vessel A	Vessel B
Diameter (mm)	4	3
Blood flow ($ml \cdot min^{-1}$)	100	100
Mean blood velocity ($cm \cdot s^{-1}$)	13.3	23.6
Shear rate (s^{-1})	13.3	31.5

2.2 Cardiovascular disease

2.2.1 Pathophysiology of atherosclerosis

Atherosclerosis is a complex process, however, it is well established that endothelial dysfunction is an early event and a precursor to atherosclerotic progression (Anderson *et al.*, 1995; Quyyumi, 2003). Traditional risk factors have been shown to diminish arterial endothelial function, potentially through reduced bioavailability of anti-atherogenic nitric oxide (NO) (Quyyumi, 2003). It is believed that the endothelium can be 'attacked' by a large range of stimuli that subsequently initiate an atherosclerotic cascade. These stimuli directly or indirectly relate to cardiovascular risk factors. For example, hypercholesterolemia increases circulating low density lipoproteins (LDL) and the rate of their infiltration past the endothelial layer (Westhuyzen, 1997). Raised levels of LDL

reduce the bioavailability of endothelium-derived NO and down-regulate endothelial eNOS, which damages the endothelium. Cigarette smoking increases oxidative free radicals (reactive oxygen species; ROS) in the blood stream (Ambrose and Barua, 2004) that also infiltrate the artery wall and bind with LDL to form oxidised LDL (ox-LDL) (Westhuyzen, 1997). Macrophages ingest ox-LDL to form foam cells and release toxic substances, including cytokines, chemokines and other enzymes which cause further damage to the endothelium and initiate an inflammatory response (Westhuyzen, 1997). Once a foam cell is formed, lipids and proliferated smooth muscle cells accumulate in the cell leading to growth of the lesion (Naghavi *et al.*, 2003a; Naghavi *et al.*, 2003b). Over time lesions can erupt, leading to downstream occlusion potentially leading to thrombosis and coronary ischemic attacks, which may deteriorate to cardiac arrest, or stroke (Naghavi *et al.*, 2003a; Naghavi *et al.*, 2003b).

2.2.2 The role of nitric oxide

In a defining experiment, Furgott and Zawadzki (1980) discovered de-endothelialised arterial smooth muscle contracted *in vitro* when treated with acetylcholine (ACh), yet paradoxically relaxed when the endothelium was left intact, suggestive of an 'endothelium derived relaxing factor' which was later identified as nitric oxide (NO) (Feelisch *et al.*, 1994). In response to increased haemodynamic flow, NO released from the endothelium regulates vascular tone through vasodilator effects. This pathway has been shown to be dependent on an undamaged endothelium in animals (Smiesko *et al.*, 1985; Pohl *et al.*, 1986) and more recently, in an *in vivo* human model (Dawson *et al.*, 2010b;a; Dawson *et al.*, 2012). This indicates that elevations in flow, and therefore shear, mediate vasodilation through a direct effect on the endothelium. Although other endothelial paracrines have been identified (Grabowski *et al.*, 1985; Busse *et al.*, 2002), NO and its vasodilator pathway is most frequently investigated due to its cardio-

protective and anti-atherogenic properties (Green *et al.*, 2004a). Nitric oxide has been shown to reduce platelet aggregation, leukocyte adhesion, formation of ox-LDL, smooth muscle proliferation and migration into foam cells (Naghavi *et al.*, 2003a).

Alongside increased risk of atherosclerosis from traditional risk factor manifestation, reduced NO bioavailability has a crucial role to play in the development of atherosclerosis. Nitric oxide is a key mediator in the vasodilator response to increased blood flow and shear stress. In response to increases in shear, endothelial nitric oxide synthase is phosphorylated before catalysing the synthesis of NO as a by-product from the reaction of L-citrulline from L-arginine (Figure 1.2) (Dimmeler and Zeiher, 2003; Hambrecht *et al.*, 2003). Rapid diffusion of NO into the smooth muscle cell (SMC) induces vasodilation induces production of cyclic guanosine monophosphate which subsequently inhibits Ca^{2+} influx into the SMC provoking hyperpolarisation and vasodilation (Figure 2.2) (Whyte and Laughlin, 2010). Other vasodilator pathways including responses to conductance and metabolites are beyond the scope of this thesis. As alluded to previously, flow-mediated vasodilation is reliant upon an intact endothelium and is therefore an endothelium dependent pathway.

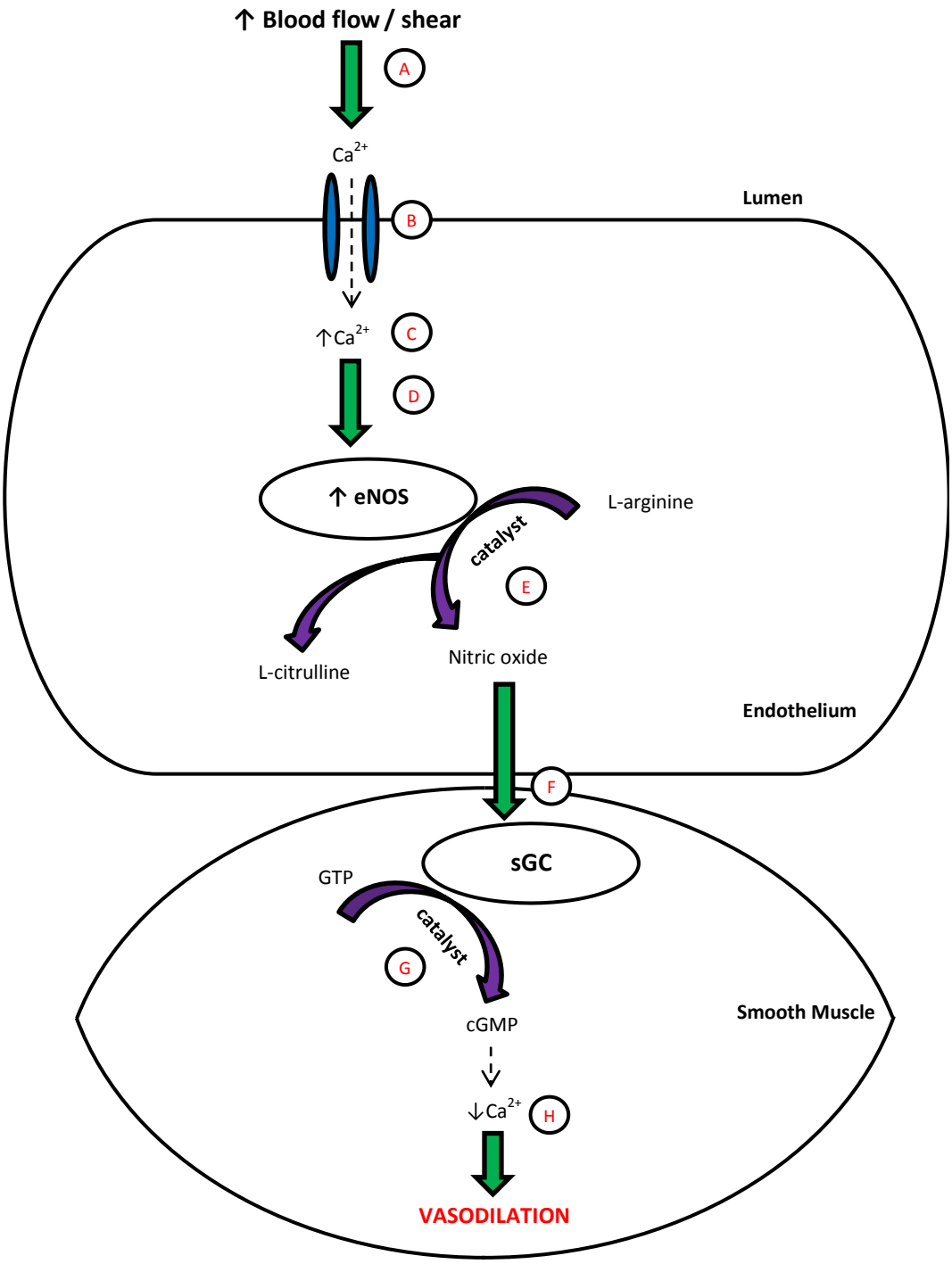


Figure 2.2. Schematic representation of nitric oxide mediated, shear stress stimulated vasodilation
A) Mechanical stimulation of stretch activated channels – morphological deformation of sac on endothelial membrane
B) Opening of ion gates – influx of Ca^{2+} into endothelial cell
C) ↑ intracellular Ca^{2+} concentration
D) Stimulation of Akt-dependent phosphorylation of eNOS on Ser^{1177} – ↑ eNOS
E) Production of nitric oxide (NO) as a by-product from eNOS catalysis of L-arginine → L-citrulline
F) Diffusion of NO across membrane → smooth muscle cell (SMC)
G) ↑ NO in the SMC stimulates production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate
H) ↑ cGMP inhibits Ca^{2+} influx into the smooth muscle cell resulting in hyperpolarisation and relaxation
Ca^{2+}, calcium; **cGMP,** cyclic guanosine monophosphate; **eNOS;** endothelial nitric oxide synthase; **GTP,** guanosine triphosphate**; sGC,** soluble guanylate cyclase

2.2.3 Treatment of endothelial dysfunction and cardiovascular disease

It is well established that physical activity is beneficial in both primary (Myers *et al.*, 2002) and secondary (Heran *et al.*, 2011) prevention of cardiovascular events. Although cardiovascular risk is reduced by ~30% through exercise alone (Thompson *et al.*, 2003), traditional risk factor amelioration is modest, attributing to only ~60% of the total benefit of exercise (Mora *et al.*, 2007). It has recently been suggested that this 'risk factor gap' could be explained by a direct cardio-protective effect of exercise on the vasculature (Green, 2009).

Episodic increases in haemodynamic flow and consequently shear stress during exercise induces secretion of NO from the endothelium. Over time this is thought to induce upregulation of endothelial NO synthase (eNOS) (Hambrecht *et al.*, 2003), positively (or outwardly) remodel arteries (Zeppilli *et al.*, 1995; Naylor *et al.*, 2006; Tinken *et al.*, 2008) and improve cardiovascular health (Niebauer and Cooke, 1996). The benefits of exercise in ameliorating endothelial dysfunction have been shown in a variety of clinical populations, including those with hypertension (Swift *et al.*, 2011), coronary artery disease (Hambrecht *et al.*, 2003), hypercholesterolemia (Walsh *et al.*, 2003), diabetes (Maiorana *et al.*, 2001), as well as in asymptomatic individuals (Clarkson *et al.*, 1999; Goto *et al.*, 2003; Dawson *et al.*, 2008).

A recent meta-epidemiological study of over 300,000 subjects highlighted that exercise is at least as effective as pharmacological intervention in secondary prevention of coronary heart disease and more effective in prevention of stroke (Naci and Ioannidis, 2013). However, the greatest gains in risk factor amelioration are intuitively found when drug and exercise interventions are combined. An example of this is cardiac rehabilitation

services which are multifaceted and include exercise, pharmacological intervention, education sessions and counselling (Bethell *et al.*, 2001; Taylor *et al.*, 2004).

2.3 Vascular adaptation

2.3.1 The role of shear stress

Shear stress, the frictional force moving blood exhibits on the endothelium, is the key stimulus for vascular adaptations. Whist there is clear evidence that exercise increases shear stress and in turn, bioavailability of NO, the pattern of shear rate (SR) through an artery can be potentially detrimental to its structure and function. In 1986, Langille and O'Donnell published a seminal paper which described a 21% reduction in rabbit common carotid artery diameter when exposed to a 70% reduction in blood flow for 2 weeks (Langille and O'Donnell, 1986). The rabbits used were euthanised before diameter measurements were made using casts and stereomicroscopes. Later studies suggested that structural changes may supersede functional changes in response to a chronic decrease in flow/shear (i.e., through physical inactivity or disease) (Silber and Sinoway, 1990; de Groot *et al.*, 2006; Thijssen *et al.*, 2010; Thijssen *et al.*, 2011b) leading to reduced artery diameter. The same is also true following an increase in flow/shear (i.e., through exercise training) which ultimately leads to positive remodelling in the coronary (Laughlin, 1995) and peripheral (Tinken *et al.*, 2008; Tinken *et al.*, 2010) vasculature.

Historically, blood was thought to continuously flow through conduit arteries in an antegrade manner. However, contemporary Doppler ultrasound blood flow velocity readings have detected that peripheral arteries are subject to oscillatory flow/SR at rest in most peripheral arteries, with portions of retrograde flow/SR during diastole (Halliwill and Minson, 2010). Increased retrograde SR has been identified as potentially detrimental to

acute conduit artery endothelial function (Thijssen *et al.*, 2009b; Tinken *et al.*, 2009; Johnson *et al.*, 2012; Schreuder *et al.*, 2014). In support of this, branched or curved sections of the vasculature and areas with high oscillatory flow, such as the lower limbs, are predisposed to atherosclerosis (Chiu and Chien, 2011).

2.3.2 Shear rate manipulation

Exploration of factors which affect shear pattern and subsequently endothelial function and cardiovascular health are in their infancy. Shear manipulation through cuff inflation (Thijssen *et al.*, 2009b; Tinken *et al.*, 2009; Johnson *et al.*, 2012; Schreuder *et al.*, 2014), lower body negative pressure (Padilla *et al.*, 2010), cold pressor tests (Padilla *et al.*, 2010) and varying exercise modalities (Thijssen *et al.*, 2009a) have given insight the potential effects of retrograde shear on endothelial function. Additionally, older individuals can exhibit almost three times the retrograde SR at rest than their healthy counterparts (Padilla *et al.*, 2011), which may constitute reason as to why asymptomatic older persons often develop atherosclerosis. The atherosclerosis prone femoral artery has significantly lower mean SR than the less susceptible brachial artery. This may suggest that the overall SR pattern (i.e., changes in both antegrade and retrograde SR) may be integral in the decline (or protection) of endothelial function and progression of atherosclerosis.

Various studies have investigated effects of SR pattern on vascular function by intentional SR manipulation. During local (handgrip) and systemic (cycling) exercise, forearm cuff inflation was shown to attenuate the expected increase in antegrade SR, which inhibited the increase in vascular function which was reported in the 'normal' non-cuffed arm (Tinken *et al.*, 2009). Furthermore, studies have shown incremental increases in retrograde shear elicited "dose"-dependent declines in both brachial (Thijssen *et al.*, 2009b; Schreuder *et al.*, 2014) and femoral (Schreuder *et al.*, 2014) vascular function.

2.3.3 Effects of oscillatory shear pattern at a cellular level

In vivo studies have revealed that oscillatory shear is detrimental to vascular function (Green *et al.*, 2003). However, they do not provide any information as to the effect of these shear patterns on the endothelium at a cellular level. *In vitro* experiments have demonstrated that endothelial cells exposed to oscillatory shear exhibit a pro-atherosclerotic phenotype (Ziegler *et al.*, 1998; Silacci *et al.*, 2001). This phenotype is characterised by increased expression of the potent vasoconstrictor endothelin-1 (ET-1) (Ziegler *et al.*, 1998), vascular cell adhesion molecule VCAM-1 (Chappell *et al.*, 1998), and intercellular adhesion molecule ICAM-1 (Hsiai *et al.*, 2001). Furthermore, studies have identified that oscillatory shear promotes smooth muscle cell proliferation (Hastings *et al.*, 2009). At the same time, exposure to oscillatory shear also leads to reduced expression of nitric oxide synthase (eNOS) (Silacci *et al.*, 2000). Taken together, *in vivo and in vitro* evidence suggests that oscillatory shear, i.e. shear patterns characterised with increased retrograde and decreased mean shear, is detrimental to vascular function and leads to a pro-atherosclerotic phenotype.

2.3.4 Shear sensing and its link to disease

Recent studies have increasing looked to investigate the link between shear sensing of the endothelium and disease. Although the mechanotransduction pathways are not fully understood, several have been proposed (Haram *et al.*, 2008). One proposed pathway involves caveolae – projections of the endothelial membrane, rich in proteins and phospholipids (Haram *et al.*, 2008). Chronic increases in shear stress have been shown to activate formation of caveolae leading to enhanced phosphorylation of eNOS and production of NO (Rizzo *et al.*, 2003). Moreover, exercise has been shown to increase the density of caveolae (Davis *et al.*, 2003). In terms of disease, disturbance of the caveolae

has been linked to increased risk of atherosclerosis (Haram *et al.*, 2008). Taken together, the caveolae system seems crucial in the upregulation of eNOS and increased production of anti-atherogenic NO. Furthermore, shear stress is the key mediator of this pathway (Haram *et al.*, 2008).

A second proposed pathway involves endothelial glycocalyx – a fragile gel barrier between the endothelial cell and the lumen (Alphonsus and Rodseth, 2014). As the most direct interface with the flowing blood, its composition constantly changes, as macromolecules are sheared by the flowing plasma (Alphonsus and Rodseth, 2014). Recent literature points to the importance of the glycocalyx in NO production. It is the first structure that flowing blood connects with which subsequently causes a mechanotransduction cascade (Pahakis *et al.*, 2007). It is postulated that pro-atherosclerotic risk factors impair glycocalyx function and that exercise / increases in shear may have a protective effect (Noble *et al.*, 2008).

2.4 Measurement of endothelial (dys)function in humans

Several methods have been adopted to assess arterial function *in vivo*. Among others, assessment of pulse pressure and its ratio to stroke volume can infer total arterial compliance, which has been cited as an independent predictor of cardiovascular events (de Simone *et al.*, 1999). Aortic pulse wave velocity, an index of arterial stiffness, has also been identified as an independent predictor of all-cause mortality and cardiovascular risk (Ben-Shlomo *et al.*, 2013). In addition, a plethora of plasma biomarkers, including highly sensitive C-reactive protein, are believed to represent endothelial function and predict future disease (Wang *et al.*, 2006).

2.4.1 Venous occlusion plethysmography

As mentioned in Chapter 1, venous occlusion plethysmography (VOP) was one of the first tools used to measure peripheral vascular function. Forearm VOP was first described as a method to measure blood flow over a century ago (Hewlett and Van Zwaluwenburg, 1909). This technique is principally used in the measurement of vascular tone in resistance vessels. It can also be used to assess the local effects of vasoactive drugs and hormones on resistance vessel tone, usually administered through the brachial artery (Wilkinson and Webb, 2001). The VOP technique is based on the principle that sub-diastolic (typically 40-60 mm Hg) cuff inflation induces venous congestion, but does not alter arterial inflow (Wilkinson and Webb, 2001). Using mercury strain-gauges, blood flow into the forearm can be estimated from changes in forearm volume. The protocol involves intermittent inflation/deflation of a pressure cuff distal to the site of measurement. It is well established practice to also exclude the hand circulation due to arterio-venous shunts and its greater skin/muscle blood flow ratio *via* inflation of a blood pressure cuff to 220 mmHg (Abramson and Ferris, 1940).

In a seminal paper, the role of NO in resistance artery vascular tone was examined by assessing changes in blood flow in response to administration of the NO inhibitor L-NMMA, *via* VOP (Vallance *et al.*, 1989). Infusion of L-NMMA induced a 50% decline in blood flow. Subsequently, endothelium dependent vasodilation through administration of acetylcholine (ACh) was attenuated; however, endothelium independent vasodilation (through administration of glyceryl trinitrate [GTN]) was not affected. This identified that NO is a mediator in both basal tone regulation and response to stimulated increases in flow (Vallance *et al.*, 1989). Assessment of endothelial function through forearm VOP is commonly performed in combination with intra-arterial infusion of endothelium

independent drugs including, sodium nitroprusside (SNP) and GTN (Creager *et al.*, 1990), as well as endothelium-dependent agonists such as ACh (Chowienczyk *et al.*, 1992). Furthermore, infusion of vasoconstrictors can also be used to assess vasomotor function (Benjamin *et al.*, 1989).

Although forearm VOP has good within-subject (Roberts *et al.*, 1986) and between-subject (Petrie *et al.*, 1998) repeatability (coefficient of variation; ~10% and ~19%, respectively), questions surrounding the basic assumptions of the protocol remain. For example, it is assumed that neither continuous inflation of the supra-systolic wrist cuff, nor intermittent inflation of the distal cuff (to 40-60 mmHg) alters arterial inflow. A study in 1989 sought to answer this question by using Doppler ultrasound to measure brachial blood flow velocity during forearm cuff inflation (Hiatt *et al.*, 1989). Velocity was measured at baseline and during three 30 second bouts of cuff inflation to 20, 40 and 60 mmHg. Despite demonstrating that velocity decreased by 26% with occlusion cuff inflation to 40 mmHg and 33% at 60 mmHg, the methodology adopted in this study was unclear and it is unknown whether the use of a wrist cuff was implemented. Moreover, 4 of the 5 participants' hands were warmed to increase flow velocity (Hiatt *et al.*, 1989). As alluded to in section 2.1.4, blood flow velocity ($cm \cdot s^{-1}$) is associated with blood flow ($ml \cdot min^{-1}$) but is not a surrogate measure. Since no diameter measurements were taken, no inference can be made as to whether flow truly was reduced during cuff inflation. Furthermore, since shear is the stimulus for arterial adaptation, and retrograde shear has been identified has detrimental to vascular health, changes in shear rate would have seemed a more appropriate outcome measure. It would also have seemed sensible for the authors to have conducted the study using an intermittent cuff inflation/deflation protocol akin to VOP. Nevertheless, the rationale behind the study remains significant since a decrease in arterial flow during forearm VOP may indicate that retrograde flow/SR

has increased. If this is the case, then repeated exposure to retrograde flow/SR in a VOP protocol may, in fact, be detrimental to vascular function.

2.4.2 Flow mediated dilation

In more recent years, the non-invasive assessment of endothelial vasomotor function *via* flow mediated dilation (FMD) has been thoroughly refined and widely popularised within the literature (Celermajer *et al.*, 1992; Corretti *et al.*, 2002; Thijssen *et al.*, 2011a). The method is based on the ability of the endothelium to release vasoactive substances in response to a marked increase in blood flow, or more specifically shear stress, which acts upon the endothelium. Typically examined in peripheral conduit arteries supplying the upper or lower limbs (ranging in diameter from 2 to 10 mm), ischaemia is induced by blocking downstream blood flow *via* inflation of a supra-systolic pressure cuff placed distally to the measurement site, around the arm or leg. Upon cuff release, a large increase in blood flow is initiated to the downstream vessels, resulting in an increase in shear stress on the conduit artery wall. The dilatory response from the conduit artery is recorded and is indicative of endothelial dys(function). This frequently used measure is endothelium-dependent (Joannides *et al.*, 1995; Green *et al.*, 2013) and is partly mediated by nitric oxide (NO) (Green *et al.*, 2013), an anti-atherogenic vasodilator (Green *et al.*, 2004a). Clinical populations with increased cardiovascular risk exhibit lower FMD responses (Maiorana *et al.*, 2001; Hambrecht *et al.*, 2003; Watts *et al.*, 2004). Furthermore, FMD has been recognised as having predictive capacity for future cardiovascular disease, although this capacity may differ according to the particular protocol (Green *et al.*, 2011) and clinical group (Ras *et al.*, 2012) studied. Finally, assessment of vascular function *via* FMD has identified that endothelial dysfunction can be detected years before clinical presentation of structural abnormalities and/or established risk factors (Celermajer *et al.*, 1992; Celermajer *et al.*, 1993; Clarkson *et al.*,

1996). Taken together, the bulk of scientific evidence indicates that FMD represents a useful tool to examine endothelial function and has independent predictive capacity for future cardiovascular disease.

2.4.3 Low-flow mediated constriction

Whilst the FMD technique is based on the ability of arteries to dilate in response to *increases* in blood flow, *'low-flow mediated constriction'* (L-FMC) relies on the ability of arteries to constrict when blood flow is *attenuated*. In 1987, Levenson and colleagues were the first to describe human brachial artery vasoconstriction *in vivo* during an acute reduction of blood flow induced by inflation of a supra-systolic wrist cuff for 60-seconds (Levenson *et al.*, 1987). This was later reinforced by Anderson and Mark (1989), who inflated a blood pressure cuff around the forearm for 10-minutes to 200 mmHg and performed measurement of the brachial artery using Doppler ultrasound. Cuff inflation induced 'circulatory arrest', a condition later renamed 'low-flow', which subsequently provoked significant vasoconstriction. The presence of a 'low-flow mediated constriction' response was observed in further experiments both in the brachial (Megnien *et al.*, 1996; Levenson *et al.*, 2001) and radial arteries (Mullen *et al.*, 2001; Spieker *et al.*, 2003).

The assessment of L-FMC was re-visited as a measure of vascular function by Gori and colleagues in 2008 (Gori *et al.*, 2008). In line with work performed 20 years earlier, the proposed method involves evaluation of the response to an acute reduction in blood flow in the radial artery. Using ultrasound, the decrease in arterial diameter is assessed after 4.5-minutes of supra-systolic cuff occlusion, where the cuff is applied distal to the placement of the probe, at the wrist. L-FMC is then calculated as the relative change (i.e., decrease) in diameter in relation to baseline resting diameter (Figure 1.1)**.**

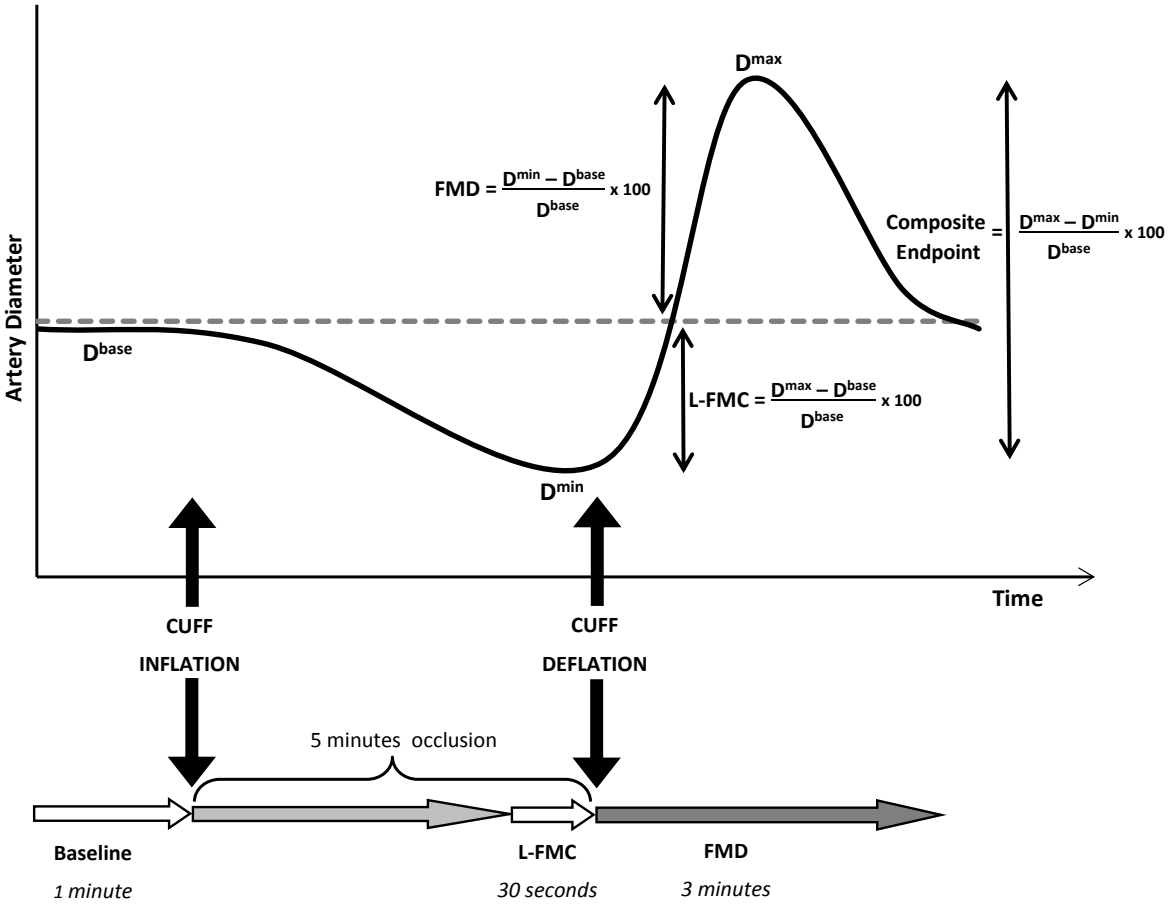


Figure 2.3. Schematic representation of concurrent L-FMC and FMD assessment in the radial artery
Reproduced from Humphreys *et al.,* (2014) with permission.
D^{base}, vessel diameter at baseline; **D^{min},** minimum vessel diameter during last 30 seconds of cuff inflation; **D^{max},** maximum vessel diameter during the first 3 minutes post cuff deflation; **FMD,** flow mediated dilation; **L-FMC,** low-flow mediated constriction; **Composite end point,** sum of L-FMC and FMD

Previous studies have argued that by focusing on dilation in response to an increase in blood flow, FMD may simplify the complexity of vasomotor control by concentrating solely on vasodilator pathways (Gori *et al.*, 2011). Low-flow mediated constriction, in contrast, examines the contraction of an artery in response to a decrease in flow.

As with FMD, the matter of reproducibility and repeatability of L-FMC is extremely pertinent. Recent large multicentre studies have highlighted that reproducible short- and medium-term FMD evaluation is attainable, so long as standardised protocols are adhered to (Ghiadoni *et al.*, 2012; Charakida *et al.*, 2013). Early, small sample L-FMC studies have revealed that L-FMC is reproducible in the brachial artery (Spiro *et al.*, 2011), and that repeatability and reproducibility of L-FMC is comparable to FMD in the radial artery (Gori *et al.*, 2008).

2.4.3.i Mechanisms of low-flow mediated constriction

Whilst *in vivo* evidence supports the belief that L-FMC is endothelium dependent (Dawson *et al.*, 2012), early blockade studies have indicated that L-FMC may not be NO mediated (Gori *et al.*, 2008). Theoretically, the mechanisms which contribute to L-FMC may include those which increase vasoconstriction and/or decrease vasodilation, although these have not yet been thoroughly examined. One mechanistic study suggested that other vasodilators which contribute to basal tone (endothelium-derived hyperpolarising factor (EDHF) and prostaglandins) may be withdrawn under conditions of low-flow (Gori *et al.*, 2008). Alternatively, L-FMC may (also) be induced by an increase in vasoconstrictor stimulus. In support of this, blockade of the potent vasoconstrictor endothelin-1 abolished the low-flow mediated constrictor response in the radial artery (Spieker *et al.*, 2003). Despite these early studies, mechanisms contributing to L-FMC remain unclear**.**

2.4.3.ii Site specificity of low-flow mediated constriction

One promising benefit of L-FMC is that it can be measured concurrently with FMD. However, initial studies utilising L-FMC were based on investigations in the radial artery, whilst the majority of FMD literature is focussed on the brachial artery. Previous studies examining *brachial* L-FMC have demonstrated differing responses to cuff inflation. Some have reported no diameter change in healthy subjects during low-flow (Filitti *et al.*, 1991; Stadler *et al.*, 1998), whilst others have found an increase (Thijssen *et al.*, 2008) or decrease (Levenson *et al.*, 2001; Spiro *et al.*, 2011) in brachial artery diameter during conditions of low-flow. Heterogeneity in responses between different arterial beds has also been demonstrated, with one study confirming the presence of L-FMC in the radial artery, whilst no such response was observed in the brachial artery of the same subjects. Despite this, evidence of vasoconstriction under conditions of low-flow in the radial artery remains (Gori *et al.*, 2008; Gori *et al.*, 2010; Weissgerber *et al.*, 2010; Dawson *et al.*, 2012; Gori *et al.*, 2012).

2.4.3.iii Low-flow mediated constriction and cardiovascular disease

L-FMC has been assessed in both healthy (Gori *et al.*, 2008; Gori *et al.*, 2010; Weissgerber *et al.*, 2010; Harrison *et al.*, 2011; Gori *et al.*, 2012; Rakobowchuk *et al.*, 2012; Rakobowchuk *et al.*, 2013) and clinical (Gori *et al.*, 2008; Gori *et al.*, 2010; Harrison *et al.*, 2011; Spiro *et al.*, 2011; Dawson *et al.*, 2012; Gori *et al.*, 2012) populations. Diminished radial artery L-FMC has been found in patients with risk factors and/or cardiovascular disease (Gori *et al.*, 2008; Gori *et al.*, 2010; Harrison *et al.*, 2011). This is usually accompanied by blunted FMD (Gori *et al.*, 2008; Gori *et al.*, 2010; Harrison *et al.*, 2011; Gori *et al.*, 2012), supporting the hypothesis that L-FMC is a marker of endothelial dysfunction. This is emphasised in a study where patients with one, two and three vessel CAD demonstrated a ‘dose-dependent’ decline in radial L-FMC (and FMD) with increased

severity of disease (Gori *et al.*, 2012). In contrast, patients assessed during acute non-ST segment elevation myocardial infarction (N-STEMI) showed greater brachial L-FMC than those with stable atherosclerosis, despite the presumption that the former had greater endothelial dysfunction and arterial disease (Spiro *et al.*, 2011). Whilst radial L-FMC responses seem to be consistently diminished in the radial artery of those with cardiovascular risk factors, both lower (Harrison *et al.*, 2011) and higher (Filitti *et al.*, 1991; Stadler *et al.*, 1998) L-FMC responses have been shown in the brachial artery, when compared to normal healthy subjects.

2.4.3.iv The impact of interventions on low-flow mediated constriction

Exercise training is a well-established non-pharmacological intervention with strong cardio-protective effects (Mora *et al.*, 2007; Green, 2009). It is generally accepted that exercise training in subjects with cardiovascular risk leads to improvement in FMD (Watts *et al.*, 2004; Maiorana *et al.*, 2011; Swift *et al.*, 2011). Similar improvements in L-FMC following exercise training might also be expected. Rakobowchuk and colleagues recently examined brachial artery L-FMC responses before and after systemic interval training in healthy subjects (Rakobowchuk *et al.*, 2012). Their 6-week protocol compared the impact of high intensity *versus* moderate intensity interval training on brachial artery L-FMC and found small, yet statistically significant, improvements in L-FMC and the composite end point following both protocols, although FMD was not significantly improved post-intervention. A plausible explanation for disparate findings between L-FMC and FMD may be that their time courses of adaptation differ.

The effect of more localised training has also been investigated *via* a 6-week handgrip training programme. Previous research has demonstrated that the radial catheterisation during angioplasty is associated with extremely impaired vascular function in the radial artery (Dawson *et al.*, 2010b). In this study (Dawson *et al.*, 2012), handgrip training was shown to recover radial artery L-FMC, FMD and the composite end point compared to impaired responses in the control group.

In other studies, the effect of acute ischemic-reperfusion injury has been investigated in the brachial artery (Rakobowchuk *et al.*, 2013; Carter *et al.*, 2014). The injury, induced *via* inflation of a cuff to 250-300 mm Hg for 20 minutes, has been shown to paradoxically augment L-FMC. This contradicts data from the radial artery, where individuals with chronic endothelial dysfunction present with diminished L-FMC (Gori *et al.*, 2008; Gori *et al.*, 2010; Gori *et al.*, 2012). With respect to chronic brachial data, some have highlighted diminished responses (Harrison *et al.*, 2011), while others have reported improvement (Filitti *et al.*, 1991; Stadler *et al.*, 1998).

2.4.3.v The composite end point

Whilst the measurement of L-FMC itself may have importance, the summative 'score' of FMD and L-FMC may provide further insight into vascular health (Gori *et al.*, 2008) (Figure 1.3)**.** The '*composite end point'* (also referred to as *'Total Vessel Reactivity'* (TVR) (Rakobowchuk *et al.*, 2012) or *'modified FMD'* (Harrison *et al.*, 2011) is indicative of constrictor/dilator reserve (i.e., the ability of a vessel to both dilate and constrict). The use of an aggregate measure in addition to FMD seems promising, although better insight into its potential clinical relevance, is essential.

2.5 Summary

This literature review focuses on some of the key effects that shear stress has upon the peripheral arteries. Studies investigating how SR manipulation has affected acute vascular function have formed an important topic of discussion. Previous studies have highlighted that cuff inflation induces an increase in retrograde SR when measuring above the position of the cuff. This has also been shown to have an acute detrimental effect on vascular function. However, it is unknown whether the SR pattern below the site of cuff inflation is altered in a similar way to that above the cuff and, furthermore, whether any change in SR would impact upon local vascular function.

This chapter has additionally highlighted other situations where cuff inflation is used, namely, during VOP. Despite VOP being an historical 'gold standard' technique for assessment of vascular function, it is based on the assumption that cuff inflation does not alter arterial inflow. Considering that continuous subdiastolic distal cuff inflation impacts upon flow and consequently, SR pattern, then it would seem plausible for intermittent cuff inflation during VOP to also alter SR pattern. Again, if this were true, then acute local vascular function may also be affected.

The present thesis aims to address this paucity of research in this area of vascular physiology. Using the novel technique of low-flow mediated constriction alongside the well-established technique of FMD, studies will identify the effect of proximal cuff inflation on vascular function, compared to distal and control conditions. Furthermore, a forearm VOP protocol will be implemented and its effect upon SR and subsequently FMD, will be investigated.

CHAPTER 3 - General methods

3.1 Experimental procedures

All experimental procedures were conducted in a temperature-controlled laboratory set to 20^{o}C. On each testing day, participants were instructed to attend in a fasted state (≥6 hours), having abstained from alcohol and caffeine (≥18 hours) and avoided vigorous exercise (≥24 hours).

On the initial testing day, participants had their height measured using a stadiometer (Model 220, Seca, Germany) and weight measured using digital scales (Model 767, Seca, Germany). Body mass index (BMI) was retrospectively calculated using the equation: [weight (kg) / height (m^2)]. On all testing days, subjects rested in a supine position for ≥15 minutes to reach physiological homeostasis. Heart rate (HR), systolic (SBP) and diastolic (DBP) arterial pressure were recorded from an automated sphygmomanometer (GE Pro 300V2, Dinamap, CA, USA) placed around the upper left arm.

3.2 Experimental techniques

3.2.1 Flow mediated dilation

Flow mediated dilation (FMD) was performed according to current methodological guidelines (Thijssen *et al.*, 2011a). Participants remained in a supine position and abducted their right arm to an ~80^{o} angle from their torso. A rapid inflation/deflation pneumatic cuff (SC-5 cuff, E-20 rapid cuff inflator; D.E Hokanson, WA, USA) was placed around the participants' upper forearm, 3-5cm below the antecubital fossa. A 10-MHz multifrequency linear array probe attached to a high-resolution ultrasound (T3000, Terason, Teratech Corp., MA, USA) was used to image the distal third of the brachial artery. Once the best longitudinal image was obtained, the B-mode image was optimised using the ultrasound software. Doppler velocity was simultaneously recorded with a

correction insonation angle of 60^{o} with the sample volume placed mid artery. Real time B-mode and Doppler velocity (pulse wave) images were recorded for 1 minute prior to rapid cuff inflation to 220 mm Hg for 5 minutes. Thirty seconds prior to cuff release and for the following 3 minutes, changes in artery diameter and blood flow velocity were recorded continuously using video recording software (Camtasia, TechSmith, MI, USA).

3.2.2 Low-flow mediated dilation

Brachial low-mediated dilation (L-FMC) was performed according to current guidelines (Gori *et al.*, 2008). Measured concurrently with FMD (as above), Doppler velocity and B-mode images during the last 30 seconds of cuff inflation were recorded. Changes in artery diameter and blood flow velocity were recorded using the same digital software.

3.3 Data analysis

3.3.1 Artery diameter, blood flow, shear rate and oscillatory shear index

Analysis of artery diameter (cm) and blood flow velocity (cm·s^{-1}) during the intervention(s) in study 1 and 2 were performed using semi-automated, custom edge-detection and wall-tracking software (intra-observer coefficient of variation of 6.7%) (Woodman *et al.*, 2001). Following successful calibration, regions of interest (ROI) were selected for analysis of diameter from the B-mode image, and of flow from the entire blood flow velocity envelope, at a frame rate of 30 Hz (Figure 3.1). Real time automatic analysis of the chosen ROI was performed in synchrony by the software. Prior training of the software to accurately detect the vessel walls was not necessary as the software is programmed to detect the wall from the brightness of the pixels. However, the ROI was carefully selected as the software is unable to accurately analyse images that are not optimised correctly. Care was taken by the sonographer when optimising the ultrasound scan images during

testing to ensure the vessel lumen was as dark as possible and the walls as bright as possible, without degrading the image. This was completed by use of the time gain, compression and noise rejection controls. The sonographer was trained to use the ultrasound for several months prior to beginning testing. Once manual selection of an ROI was made the software analysed the images automatically. Shear rate (s^{-1}) data were calculated by the analysis program based on the estimated equation of: [4 x Mean Blood Velocity ($cm \cdot s^{-1}$) / Vessel Diameter (cm)], and stored for interpretation and further analysis. Blood flow was also calculated by the software based on the equation: Blood Flow ($ml \cdot min^{-1}$) = Mean Blood Velocity ($cm \cdot min^{-1}$) x Vessel Cross Sectional Area (cm^2). Oscillatory shear index (OSI), a dimensionless parameter with arbitrary units used to indicate the magnitude of oscillation, was calculated retrospectively based on the equation: (Retrograde Shear / Antegrade Shear + Retrograde Shear) using absolute values (Newcomer *et al.*, 2008). An OSI of zero represents unidirectional antegrade flow whereas an OSI of 0.5 represents pure oscillation i.e., equal amounts of antegrade and retrograde flow (Padilla *et al.*, 2010). Diameter measurements are presented in mm.

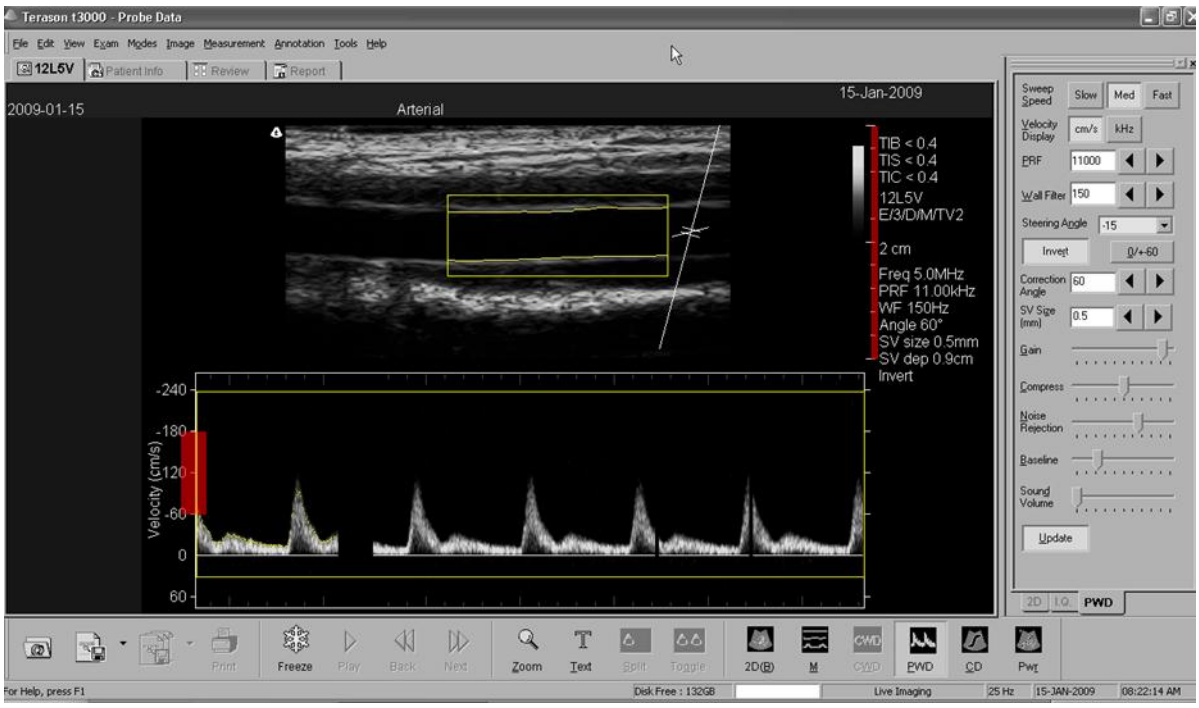


Figure 3.1. Screen shot of analysis depicting selection of regions of interest
Regions of interest (yellow boxes), calibration of blood flow velocity (cm·s^{-1}) and diameter (cm) (red boxes)

3.3.2 Flow mediated dilation, low-flow mediated constriction and composite end point

Briefly, blood flow velocity and diameter were recorded for 60 seconds prior to cuff inflation and then continuously for 30 seconds before and 3 minutes after cuff release. The semi-automated software analysed diameter and blood flow velocity and calculated SR in real time from selected ROI (Figure 3.2). Outputs of baseline diameter (mm), peak diameter (mm), relative change in diameter (% change from baseline) and time to peak dilation (TTP, s) were calculated automatically by the software for FMD. Additionally, the FMD shear rate area under the curve (SR_{AUC}) response was calculated as the shear rate stimulus from cuff release to peak dilation. Outputs of baseline diameter (mm) and minimum diameter (mm) were calculated automatically by the software for L-FMC. Manual calculation of the % change in diameter from baseline was then performed. Mean shear rate (s^{-1}) was also calculated during the last 30 seconds of cuff inflation to quantify the low-flow stimulus. The composite end point (sum total of L-FMC and FMD using absolute values) was manually calculated retrospectively.

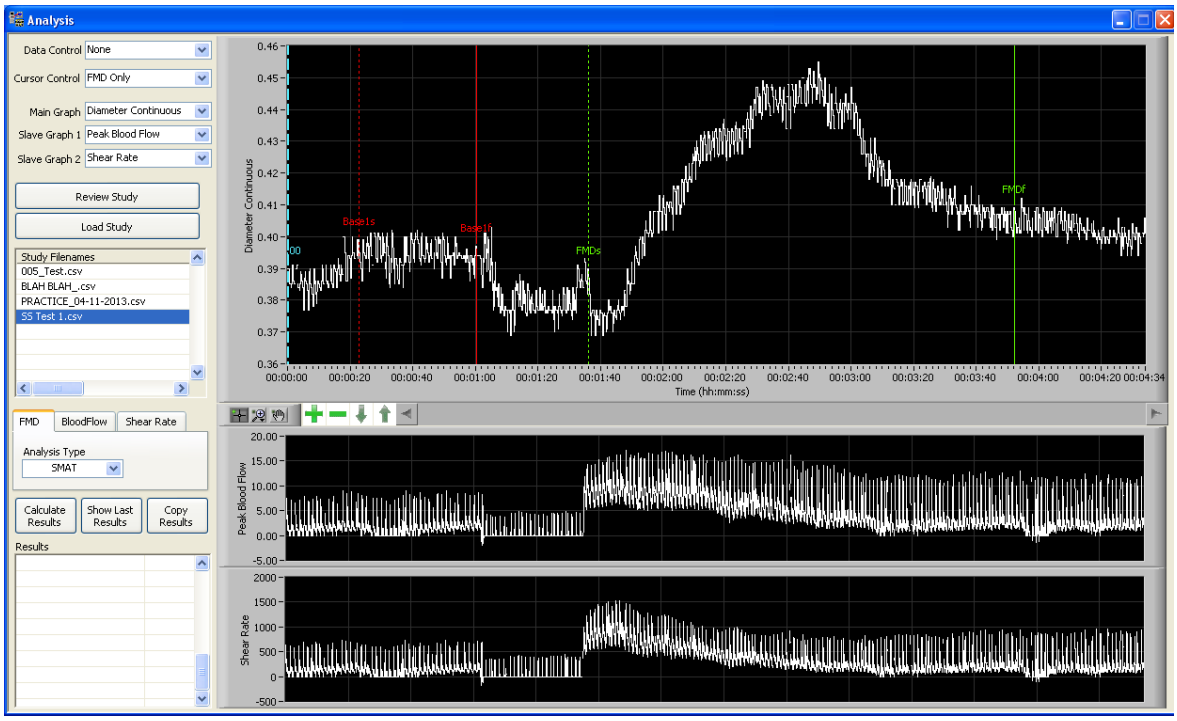


Figure 3.2. Screen shot of analysis output
Chosen baseline (Base1s – Base1f), FMD start (FMDs) and FMD finish (FMDf)

3.3.3 Frame analysis and quality control

All scans and subsequent analysis were performed by the same user who was not blinded. However, during real time automatic analysis of diameter and blood flow velocity changes by the software, care was taken to take note of any sections of scan where the automated software did not correctly identify the vessel wall. This was done through visual inspection of the ROI during the scan. Manual deletion of these sections was completed by the user. Sections could be deleted with frame-by-frame precision. This was especially important during baseline measurement and around peak dilation, as errors here would have invalidated the percentage change in FMD.

The decision to analyse L-FMC as well as FMD was taken retrospectively. Low-flow mediated constriction was only analysed in scans where the same ROI as used for FMD was of reliable quality for assessment of L-FMC. All scans where this was not possible (including scans where another ROI could have been used) were not included. Therefore n=13 for L-FMC data in Study 1.

3.4 Statistical analysis

Data are presented as mean ± SD unless otherwise stated. Alpha significance level was set at $P<0.05$. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) for Windows (Version 20, SPSS Inc., Chicago, IL, USA). Pairwise comparisons were performed when significant main or interaction effects were detected. Fisher's least significant difference (LSD) correction was used to correct for multiple comparisons in line with advice for repeated measures analysis (Perneger, 1998). Details of specific statistical tests used can be found in each chapter.

CHAPTER 4 – Study 1
The influence of proximal cuff inflation on shear rate pattern and vascular function

4.1 Introduction

Shear rate (SR), the drag force that moving blood conveys to the endothelium, is a stimulus for vascular adaption. Whilst episodic increases in SR (e.g., through exercise) promotes improvement in vascular function and positive remodelling of conduit arteries (Tinken *et al.*, 2008), leading to improved cardiovascular health (Niebauer and Cooke, 1996; Green, 2009), some SR patterns can have a negative effect upon vascular function and health. Acute increases in retrograde shear rate (SR) have been shown to diminish vascular function *in vivo* (Thijssen et al., 2009b; Johnson et al., 2012). Furthermore, repeated oscillatory shear patterns (characterised by increased retrograde and decreased mean SR) may be pro-atherogenic (Ziegler *et al.*, 1998; Silacci *et al.*, 2001).

Previous studies have used cuff inflation techniques to manipulate SR *in vivo* in an attempt to further understand the acute effects of increased retrograde SR on vascular function, as measured by FMD. In these studies, SR manipulation has been conducted by inflation of a cuff distal to the site of measurement (Thijssen *et al.*, 2009b; Tinken *et al.*, 2009; Schreuder *et al.*, 2014). It is unknown, however, whether a cuff inflated proximal to the site of measurement would induce a different SR pattern and whether this would have a different effect on FMD.

The primary purpose of this present study is to investigate the effects of 30 minutes of sub-diastolic distal and proximal cuff inflation on FMD in the brachial artery. The hypothesis for this study is that proximal cuff inflation will alter SR pattern in the mid brachial artery to a similar degree as distal cuff inflation and that FMD will be attenuated in both conditions, compared with control.

4.2 Methods

4.2.1 Sample size estimation

A sample size estimation was conducted based on data from a previous study which used cuff inflation to manipulate shear (Thijssen *et al.*, 2009b). The primary outcome was change in FMD from baseline to post cuff occlusion within subjects. Using the statistical package Minitab (version 16) it was estimated that 19 subjects would achieve adequate statistical power (80%) to detect a statistically significant difference of 3.0% in FMD (α significance level of 0.05) with a standard deviation of 3.2, using a two-sample *t*-test.

4.2.2 Participants

Nineteen young healthy males were recruited and screened for this within-subjects, 3 condition, randomised trial. Exclusion criteria included: females, males <18 and >45 years, smokers, and those with known cardiovascular disease. One participant was excluded due to hypertension (blood pressure >140/80). The remaining 18 subjects (age 27.3 ± 5.1 yr, height 1.81 ± 0.07 m, weight 78.10 ± 12.15 kg, BMI 23.82 ± 3.02 kg/m^2) completed all 3 trials. Due to technical difficulties, L-FMC was only analysable in 13 subjects. The study procedures were approved by the ethics committee of Liverpool John Moores University and adhered to the Declaration of Helsinki. Informed written consent was obtained from all participants prior to experimental testing.

4.2.3 Experimental design

Participants attended the laboratory on three separate occasions that were separated by ≥24 hours. Testing was completed at the same time of day to avoid circadian variation on physiological outcome measures. Flow mediated dilation (FMD) and low-flow mediated constriction (L-FMC) were assessed in the right brachial artery before and immediately after 30 minutes of shear rate (SR) manipulation. Manipulation was performed in a randomised order by inflating a cuff to 60 mm Hg either below (distal) or above (proximal) the mid brachial artery, in comparison with a no cuff (control) condition (Figure 4.1).

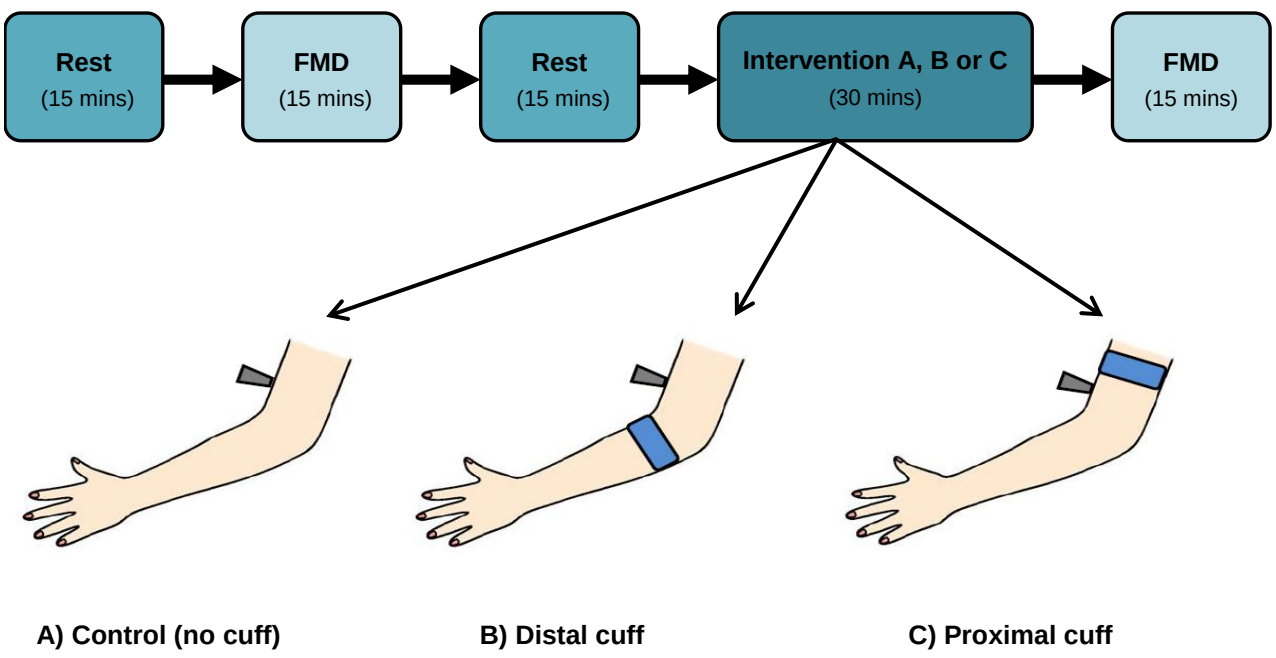


Figure 4.1. Study 1 experimental design

4.2.4 Experimental procedures

On all testing days, subjects rested in a supine position for ≥15 minutes to reach physiological homeostasis. Heart rate (HR), systolic (SBP) and diastolic (DBP) arterial pressure were recorded from an automated sphygmomanometer placed around the upper left arm. Subsequently, L-FMC and endothelium dependent NO-mediated vascular function (FMD) were examined in the right brachial artery according to current guidelines (Gori *et al.*, 2008; Thijssen *et al.*, 2011a)

Following an additional 15 minutes of supine rest post initial L-FMC/FMD, baseline diameter and blood flow velocity were recorded immediately before the 30 minute intervention. During the intervention, subjects received one of the following conditions in a randomised order: 1) cuff inflation (60 mm Hg) distal to the brachial artery measurement site, 2) cuff inflation (60 mm Hg) proximal to the brachial artery measurement site and 3) a no cuff control condition. Artery diameter and blood flow velocity were recorded continuously for 60 seconds at 5 minute intervals. The ultrasound probe remained in the same position throughout the entire 30 minute intervention. After 25 minutes, blood pressure and heart rate were again recorded. Immediately following the 30 minute intervention, L-FMC/FMD was repeated. All testing sessions were completed by the same sonographer.

4.2.5 Data analysis

Full details of all data analysis for FMD, L-FMC, composite end point, artery diameter, shear rate and oscillatory shear index can be found in section 3.2.

4.2.6 Statistical analysis

4.2.6.i Baseline data

Baseline SBP, DBP, heart rate (HR) and shear data were analysed using one-factor (condition) linear mixed models (LMMs).

4.2.6.ii Blood pressure, heart rate, shear rate, diameter and oscillatory shear index data

Changes in SBP, DBP, HR, shear rate and diameter data were analysed using two-factor (3 condition x 2 time) LMMs. Specifically, shear and OSI were initially analysed as the average of the 6 intervention time points (5, 10, 15, 20, 25 and 30 minutes) compared to baseline using two-factor LMMs (3 condition x 2 time). Subsequently, more detailed analyses at each time point were conducted using two-factor LMMs (3 condition x 7 time). Flow mediated dilation and L-FMC data (baseline diameter, minimum diameter, peak diameter, %FMD, TTP, SR_{AUC} and mean SR during cuff inflation) were also analysed using two-factor LMMs.

4.2.6.iii Flow mediated dilation data

Additionally, the FMD response was analysed according to allometric modelling (Atkinson *et al.*, 2013). Briefly, baseline and peak diameter were log transformed (into logDbase and logDpeak, respectively) and the difference (logDdiff FMD) was calculated. A two-factor LMM was performed with logDdiff as the dependent variable and logDbase and $logSR_{AUC}$ as covariates. These covariates were chosen since it has been demonstrated that FMD is inversely correlated to baseline diameter, and that SR_{AUC} is also inversely correlated with baseline diameter and controlling for both has been suggested as the preferred method of FMD analysis (Atkinson and Batterham, 2013). Mean logDdiff FMD values were subsequently anti-logged back to equivalent adjusted FMD% values.

4.2.6.iv Low-flow mediated constriction and composite end point data

It has been suggested that L-FMC may also vary in magnitude depending on baseline diameter (Humphreys *et al.*, 2014). As such, covariate control has also been implemented in the statistical analysis of L-FMC. The same method was also used for L-FMC response with baseline and minimum diameter log transformed and logDdiff L-FMC calculated. A two-factor LMM was also performed with only logDbase covariate control since SR_{AUC} data is not applicable to L-FMC. Mean logDdiff L-FMC values were anti-logged back to adjusted L-FMC% values. A similar method was used for the composite end point by calculation of logComposite via the absolute sum of logDdiff FMD and logDdiff L-FMC. Again, a two-factor LMM was performed with logComopsite as the dependent variable and logDbase covariate control. All FMD, L-FMC and composite end point values are presented as adjusted % values in the text, tables and figures unless otherwise stated.

4.2.6.v Artery constriction during supra-systolic cuff inflation

A three-factor LMM (condition x time [pre vs. post] x diameter [baseline vs. minimum]) was performed in order to identify whether constriction during the assessment of L-FMC was significant compared to baseline diameter.

4.3 Results

4.3.1 Effect of cuff position on blood pressure, shear rate and oscillatory shear index

There were no significant differences in SBP, DBP, HR (Table 4.1), shear or OSI (Table 4.2) between the 3 conditions at baseline (1-factor LMM; main effect of condition $P \geq 0.7$ in all parameters).

There were no significant changes in SBP or DBP between conditions or over time. Despite no significant main effect of condition or interaction for HR, a significant main effect of time ($P=0.007$) was present. Pairwise comparisons revealed that HR dropped by a similar amount in all 3 conditions (Table 4.1).

Table 4.1. Blood pressure and heart rate before and during each condition (n=18)

	Control		Distal		Proximal		LMM *P*		
Parameter	Baseline	Intervention	Baseline	Intervention	Baseline	Intervention	Condition	Time	C. x T.
SBP, mm Hg	119 ± 9	119 ± 9	118 ± 9	116 ± 9	119 ± 9	120 ± 9	0.294	0.665	0.593
DBP, mm Hg	66 ± 5	68 ± 5	67 ± 5	66 ± 5	66 ± 5	68 ± 5	0.682	0.156	0.188
HR, bpm	56 ± 11	54 ± 11	55 ± 11	53 ± 11	56 ± 11	53 ± 11	0.720	0.007	0.997

P-values refer to two-factor LMM. No significant interactions were detected. A significant effect of time was detected for HR.
DBP, diastolic blood pressure; **HR**, heart rate; **SBP**, systolic blood pressure.

Both proximal and distal cuff positions significantly altered SR pattern over time, whilst such changes in SR pattern did not occur during the control condition (Figure 4.2; Table 4.2). Following detection of significant main effects (time and condition) and a significant interaction, pairwise comparisons identified retrograde SR was significantly increased by both distal and proximal cuff interventions ($P<0.0005$; Table 4.2). Distal cuff inflation induced significantly greater retrograde SR than the proximal intervention ($P=0.003$). Only a main effect of time was present for antegrade SR, with post-hoc analysis identifying a significant decrease during proximal cuff inflation ($P<0.0005$; Table 4.2) but not during distal inflation ($P=0.624$; Table 4.2) (Figure 4.2).

Significant main effects (condition and time) as well as an overall significant interaction were found for OSI (P<0.0005). There was no significant change in OSI over time in the control condition, whereas in both distal and proximal conditions, OSI increased significantly from baseline (P<0.0005; Table 4.2). Notably, despite different SR patterns resulting from distal and proximal cuff inflation, the overall change in OSI during cuff inflation was not significantly different between distal and proximal conditions (P=0.486; Table 4.2).

In order to identify any normalisation or compensatory effects of SR and OSI over time, statistical analysis of SR and OSI at all 7 time points throughout each intervention was conducted using a two-factor LMM. Pairwise comparisons highlighted significant changes in SR pattern and OSI following cuff inflation occurred within the first 5 minutes in both proximal and distal conditions. These changes in mean, antegrade and retrograde SR, and OSI were persistent throughout each 30 minute intervention (Figure 4.3).

Table 4.2. Shear rate and blood flow velocity characteristics before and during each condition (n=18)

	Control		Distal		Proximal		LMM *P*		
Parameter	Baseline	Intervention	Baseline	Intervention	Baseline	Intervention	Condition	Time	C. x T.
Mean SR, s^{-1}	76 ± 48	78 ± 48	69 ± 48	36 ± 48*†	74 ± 48	31 ± 48*†	<0.0005	<0.0005	0.004
Antegrade SR, s^{-1}	89 ± 44	88 ± 44	83 ± 44	79 ± 44#	89 ± 44	62 ± 44*†	0.066	0.025	0.056
Retrograde SR, s^{-1}	-13 ± 14	-10 ± 14	-14 ± 14	-43 ± 14*†#	-14 ± 14	-31 ± 14*†	<0.0005	<0.0005	<0.0005
OSI, a.u	0.15 ± 0.11	0.13 ± 0.11	0.17 ± 0.11	0.35 ± 0.11*†	0.16 ± 0.11	0.33 ± 0.11*†	<0.0005	<0.0005	<0.0005
BF velocity ($cm \cdot s^{-1}$)	7.4 ± 4.4	7.6 ± 4.4	6.6 ± 4.4	3.3 ± 4.4*†	7.3 ± 4.4	3.0 ± 4.4*†	<0.0005	<0.0005	0.002

P-values refer to two-factor LMM. Significant main effects and interactions were detected in mean SR, retrograde SR, OSI. A significant main effect of time only was detected in antegrade SR. **a.u**, arbitrary units; **avg.**, average; **BF,** blood flow; **OSI**, oscillatory shear index; **SR**, shear rate.
Pairwise comparisons: *significantly different from baseline; †significantly different from control condition, #significantly different from proximal condition (*P*<0.05).

Table 4.3. Flow mediated dilation of healthy subjects before and after each condition (n=18)

	Control		Distal		Proximal		LMM *P*		
Parameter	Pre	Post	Pre	Post	Pre	Post	Condition	Time	C. x T.
Baseline D, mm	3.98 ± 0.04	3.97 ± 0.04	4.00 ± 0.04	3.95 ± 0.04	3.96 ± 0.04	3.95 ± 0.04	0.803	0.518	0.788
Peak D, mm	4.23 ± 0.04	4.25 ± 0.04	4.27 ± 0.04	4.18 ± 0.04	4.20 ± 0.04	4.23 ± 0.04	0.853	0.624	0.293
FMD, %	6.67 ± 2.55	7.08 ± 2.55	6.72 ± 2.55	5.80 ± 2.55	6.29 ± 2.55	7.18 ± 2.55	0.283	0.689	0.069
Adj. FMD, %	6.61 ± 2.16	6.93 ± 2.15	6.82 ± 2.15	5.76 ± 2.15*	6.29 ± 2.15	7.14 ± 2.16	0.385	0.743	0.043
TTP, s^{-1}	53.6 ± 17.6	50.3 ± 17.6	43.8 ± 17.6	44.8 ± 17.6	46.4 ± 17.6	39.2 ± 17.6†	0.024	0.274	0.524
SR_{AUC}, 10^4	21.9 ± 9.5	19.8 ± 9.5	18.2 ± 9.5	18.2 ± 9.5	19.3 ± 9.5	17.9 ± 9.5	0.154	0.315	0.766

P-values refer to two-factor LMM. A significant interaction was detected in Adj. FMD. A significant main effect of condition was detected in TTP.
Adj, FMD, adjusted flow mediated dilation; **a.u**, arbitrary units; **SR_{AUC},** shear rate area under the curve; **D**, diameter; **FMD**, flow mediated dilation; **TTP**, time to peak. Pairwise comparisons: *significantly lower than control and proximal conditions, †significantly different from control condition

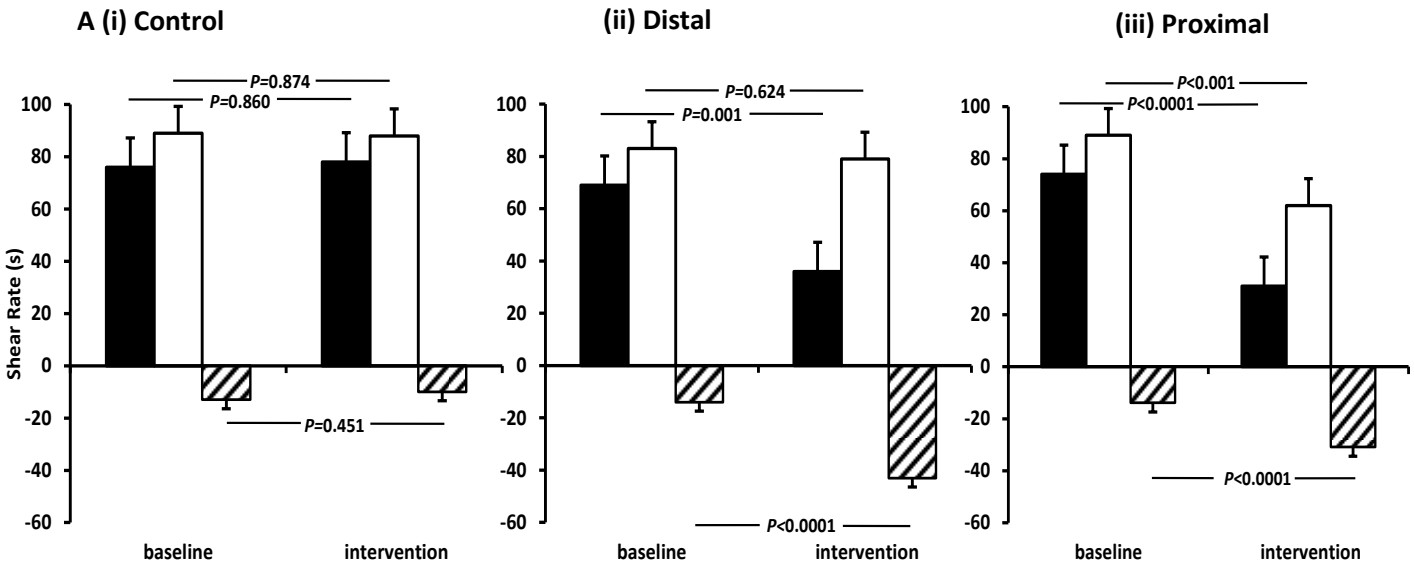


Figure 4.2.A. Mean ■, antegrade □ and retrograde ▨ shear rate at baseline and during each condition (average 5-30 minutes); within condition representation

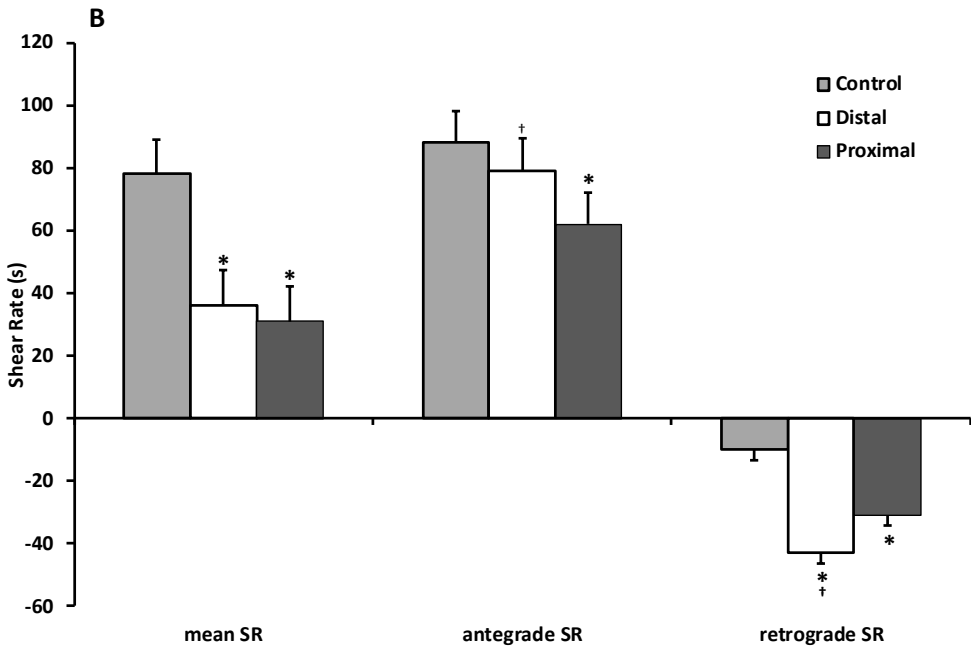


Figure 4.2.B Mean, antegrade and retrograde SR during each condition (average 5-30 minutes); between condition representation

Pairwise comparisons: *significantly different from control condition, †significantly different from proximal condition (P<0.05). Error bars represent SE.

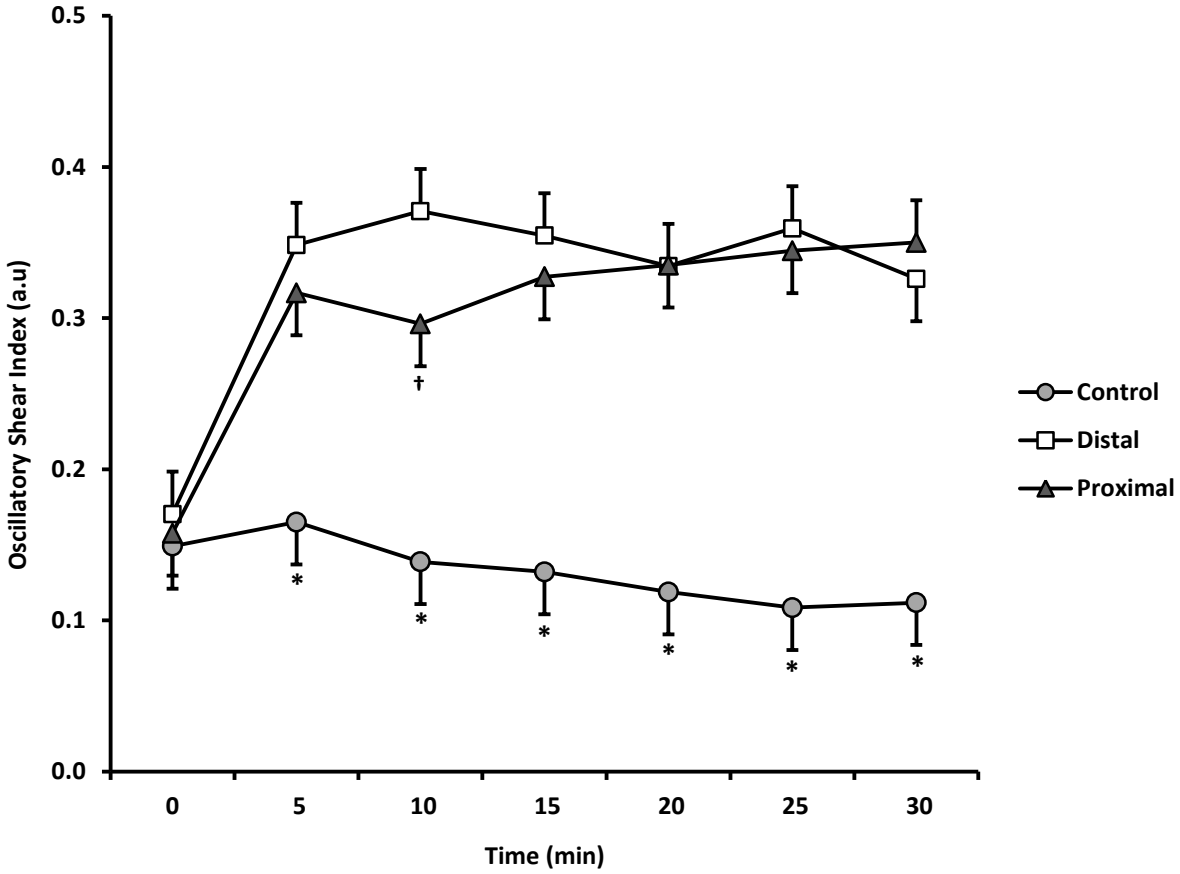


Figure 4.3. Oscillatory shear index at 0 to 30 minutes during each intervention
Pairwise comparisons: *significantly different from distal and proximal conditions, †significantly different from distal condition (P<0.05). Error bars represent SE.

4.3.2 Effect of cuff position on vessel diameter and flow mediated dilation

Baseline diameter, peak diameter, FMD%, adjusted FMD% were all similar between conditions at baseline (1-factor LMM; P≥0.6). Although not significantly different from each other, TTP and SR_{AUC} were less similar at baseline than the aforementioned variables (1-factor LMM; P≥0.2).

Artery diameter was not affected by 30 minutes of proximal or distal cuff inflation as evidenced by no significant changes in diameter between conditions or over time (P≥0.518; Table 4.3).

Despite no main effect of condition or time, there was a significant interaction for adjusted FMD% (*P*=0.043; Table 4.3). There was a trend that distal cuff inflation decreased adjusted FMD%, although this did not reach statistical significance (*P*=0.071; Table 4.3; Figure 4.4). Proximal inflation increased adjusted FMD%, but again, this change did not reach level of significance (*P*=0.097; Table 4.3; Figure 4.4). Immediately following 30 minutes of cuff inflation, adjusted FMD% was significantly lower in the distal condition than both proximal and control conditions (*P*=0.013 and *P*=0.033, respectively). There was no significant difference after the intervention between proximal cuff inflation and control (*P*=0.704). When the analysis of FMD% was performed without adjustment for baseline and SR_{AUC}, no significant differences were detected (Table 4.3).

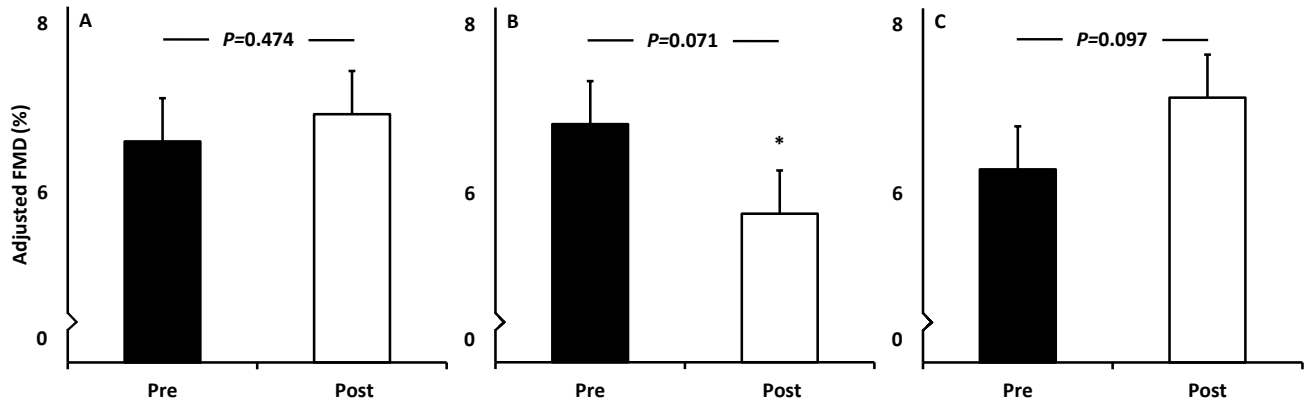


Figure 4.4. Flow mediated dilation pre- and post- intervention
A, control; **B**, distal; **C**, proximal. **FMD,** Flow mediated dilation.
Pairwise comparisons: *significantly different from control and proximal conditions. Error bars represent SE.

4.3.3 Effect of cuff position on low-flow mediated constriction and composite end point

No variables were significantly different at baseline (1-factor LMM; $P \geq 0.2$). Due to imaging issues, only data from 13 of the original participants were analysed for L-FMC and the composite end point (sum total of L-FMC and FMD).

Analysis of the 13 participants' data showed no significant main effects (condition and time) or interaction in any parameters other than adjusted FMD% and non-adjusted FMD% (Table 4.4, Figure 4.5). Cuff inflation did not significantly alter L-FMC or the composite end point.

4.3.4 Artery constriction during supra-systolic cuff inflation

The name 'low-flow mediated constriction' can be misleading since an artery does not always constrict under conditions of low-flow (i.e., cuff inflation). Therefore, a three-factor LMM [condition x time x (baseline vs. minimum diameter)] was performed in order to identify whether any constriction during the assessment of L-FMC was significant compared to baseline diameter. Minimum artery diameter was not significantly different from baseline in any condition, pre or post intervention ($P \geq 0.3$; Table 4.4), highlighting that any constriction was of negligible magnitude.

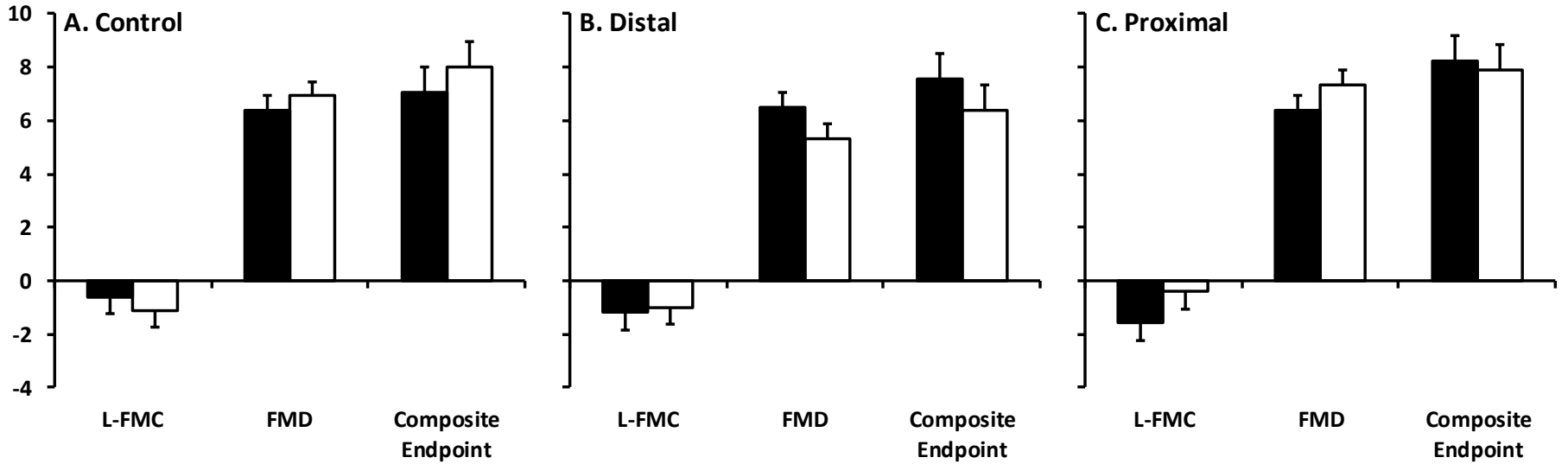
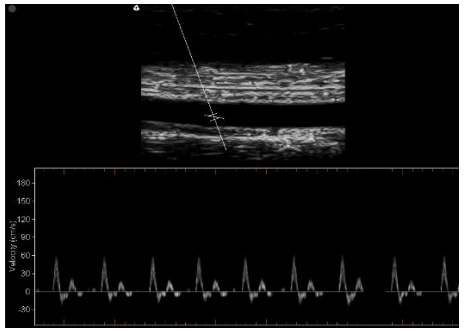


Figure 4.5. Low-flow mediated constriction, flow mediated dilation and composite end point pre- and post- intervention
FMD, Flow mediated dilation; **L-FMC,** Low-flow mediated constriction. Error bars represent SE.

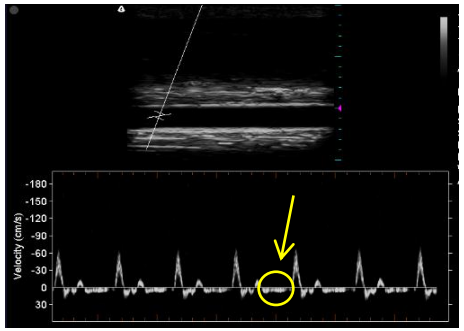
Table 4.4. Brachial flow mediated dilation of healthy subjects before and after each condition (n=13)

	Control		Distal		Proximal		LMM *P*		
Parameter	Pre	Post	Pre	Post	Pre	Post	Condition	Time	C. x T.
Baseline D, mm	4.03 ± 0.04	4.00 ± 0.04	3.95 ± 0.04	3.96 ± 0.04	4.01 ± 0.04	3.98 ± 0.04	0.829	0.874	0.966
Min D, mm	3.92 ± 0.04	3.89 ± 0.04	3.92 ± 0.04	3.89 ± 0.04	3.83 ± 0.04	3.87 ± 0.04	0.173	0.806	0.577
Peak D, mm	4.19 ± 0.04	4.21 ± 0.04	4.23 ± 0.04	4.14 ± 0.04	4.14 ± 0.04	4.17 ± 0.04	0.575	0.652	0.397
L-FMC, %	-0.65 ± 2.26	-1.10 ± 2.26	-1.19 ± 2.26	-0.93 ± 2.26	-1.47 ± 2.26	-0.33 ± 2.26	0.947	0.542	0.452
Adj. L-FMC, %	-0.60 ± 2.29	-1.09 ± 2.29	-1.19 ± 2.29	-1.00 ± 2.29	-1.59 ± 2.29	-0.40 ± 2.29	0.956	0.569	0.419
FMD, %	6.41 ± 2.23	6.90 ± 2.23	6.44 ± 2.23	5.37 ± 2.23†	6.54 ± 2.23	7.43 ± 2.23	0.025	0.747	0.037
Adj. FMD, %	6.40 ± 1.96	6.93 ± 1.96	6.50 ± 1.96	5.34 ± 1.96*†	6.40 ± 1.96	7.36 ± 1.96	0.053	0.841	0.031
Comp. E, %	7.05 ± 3.15	8.00 ± 3.15	7.64 ± 3.15	6.30 ± 3.15	8.00 ± 3.15	7.77 ± 3.15	0.372	0.697	0.224
Adj. Comp. E, %	7.04 ± 3.44	8.00 ± 3.43	7.57 ± 3.44	6.40 ± 3.43	8.22 ± 3.44	7.90 ± 3.44	0.733	0.244	0.125
Mean SR, s^{-1}	11 ± 19	26 ± 19	16 ± 19	14 ± 19	7 ± 19	9 ± 19	0.066	0.136	0.130

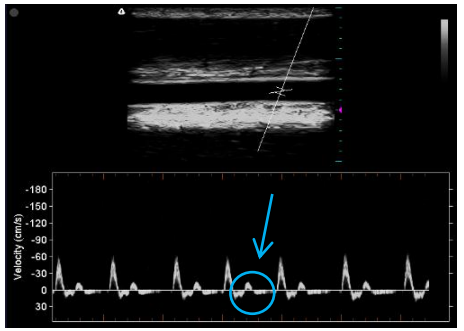
P-values refer to two-factor LLM. A significant main effect of condition was detected in FMD. Significant interactions were detected in FMD and Adj, FMD. **Adj. Comp. E,** adjusted composite end point; **Adj. FMD**, adjusted flow mediated dilation; **Comp. E,** Composite end point, **D**, diameter; **FMD**, flow mediated dilation, **SR,** shear rate. Pairwise comparisons: *significantly different from baseline P=0.043 ; †significantly different from control and proximal conditions $P \leq 0.008$.



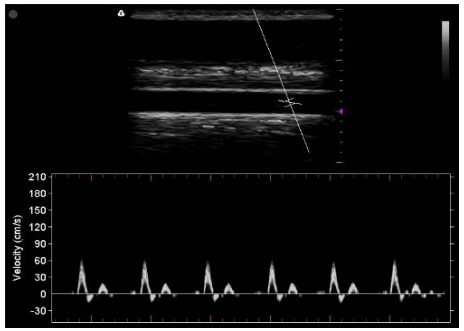
Subject 1 Control



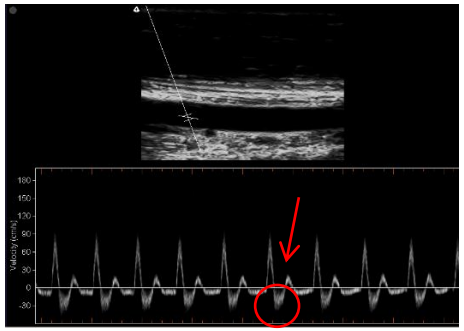
Subject 1 Distal



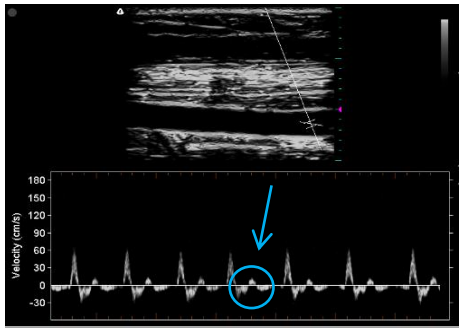
Subject 1 Proximal



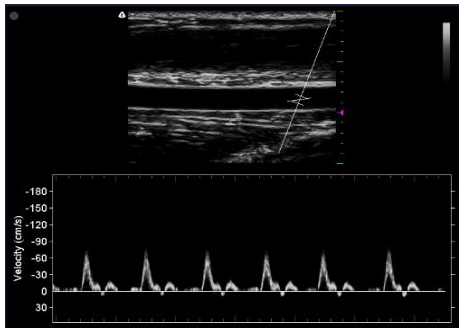
Subject 2 Control



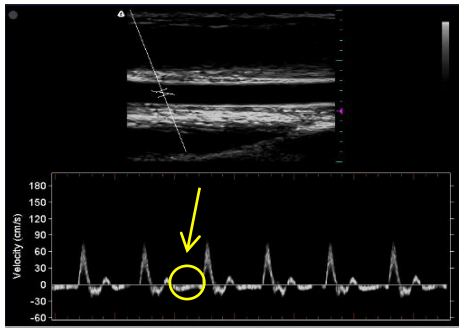
Subject 2 Distal



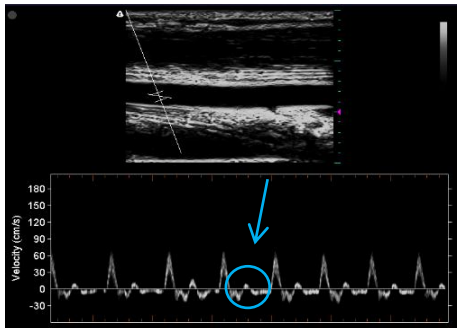
Subject 2 Proximal



Subject 3 Control



Subject 3 Distal



Subject 3 Proximal

Figure 4.6. Case study of waveform patterns during each condition for 3 subjects

4.3.5 Case study of waveforms during each condition

In a small case study of 3 participants, waveforms during each condition were visually analysed (Figure 4.6). Waveforms were similar in all 3 subjects during the control condition, with high resistance normal triphasic waveform noted. During the distal condition, waveforms of two participants were similar, with more pronounced retrograde blood flow velocity during late systole and more notably, prolonged retrograde blood flow velocity during mid-late diastole (yellow circles), compared with control. The other participant (subject 2) had a much greater increase in the magnitude of retrograde blood flow velocity during late systole (red circle). The waveforms of all 3 subjects during proximal cuff inflation were similar, with more pronounced retrograde blood flow velocity during late systole and more and prolonged retrograde blood flow velocity during mid diastole (blue circles), compared with control. Of note, two of the distal waveforms (yellow circles only) and all of the proximal waveforms (blue circles) were similar; with the only difference being that distal cuff inflation seemed to induce retrograde flow throughout the whole of mid-end diastole.

4.4 Discussion

The purpose of the present study was to examine the effect of cuff placement proximal to the site of measurement, on shear rate (SR) pattern and to determine whether this would affect vascular function assessed *via* flow mediated dilation (FMD). It was hypothesised that since SR is altered above the site of cuff inflation, that a similar change would take place below the site of cuff inflation. This is the first study to manipulate retrograde and antegrade shear using both proximal and distal occluding cuffs, within subjects. The principle novel finding was that both proximal and distal cuff inflation induced a significant increase in retrograde SR, with distal placement causing the greatest change from baseline. In both conditions, mean shear was significantly decreased from baseline when compared with the control condition, but were of similar magnitude to each other. FMD was found to be significantly lower following 30 minutes of distal cuff inflation which confirms previous research (Thijssen *et al.*, 2009b; Schreuder *et al.*, 2014). More importantly, despite a significant increase in retrograde SR as a result of proximal cuff placement (albeit not to the same degree as distal cuff occlusion), there was no significant change in FMD. This would lead one to believe that factors other than an increase in retrograde SR under proximal cuff inflation may have contributed to the finding of no acute change in vascular function following cuff inflation.

There are numerous factors that might have contributed to the potential 'protection' of vascular function under 30 minutes of proximal cuff inflation. However, due to the non-invasive nature of the present study, the exact underlying mechanisms are yet to be fully elucidated. Although both experimental conditions induced significantly greater SR than the control condition, there was significantly greater retrograde SR after distal cuff

inflation than proximal cuff. Based on the dose-response relation between acute changes in retrograde shear rate and impairment in endothelial function (Thijssen *et al.*, 2009b), a difference in the level of increment in retrograde shear rate may explain why FMD was not impaired after the proximal cuff inflation. Hence our findings reinforce previous observations, that larger increases in retrograde shear induce larger detrimental effects on FMD. It is interesting to note that, despite similar overall oscillatory shear index (OSI) between both distal and proximal experimental conditions, FMD was only significantly impaired as a result of the distal cuff position, in which the retrograde shear was larger. When visually analysing the waveforms of 3 participants during each condition (Figure 4.6, two subjects had very similar waveform patterns during both distal and proximal cuff inflation. However, one subject had very different patterns between conditions. This highlights that the response to cuff inflation between participants is individual and not uniform, making identification of trends in waveforms extremely difficult. Indeed, it has been suggested that muscle sympathetic activation and blood pressure can acutely affect shear pattern, which may explain some of the between-subject variation (Padilla *et al.*, 2010).

In addition to the effect of cuff position on SR, cuff inflation may have had other effects. One potential explanation for the difference in FMD change between distal and proximal conditions pertains to the influence of acute changes in localised pressure on endothelium function. A recent review speculated about the effect of acute changes in pressure on vascular function (Newcomer *et al.*, 2011). Reference was made to the rhythmic stretching of the elastic arteries in response to the change in pressure throughout each cardiac cycle, called cyclic strain. It is possible that proximal cuff inflation

may have affected local pressure which, in turn, may have directly impacted the local function in the brachial artery. Blood pressure measurements were taken in the contralateral arm and, as such, are representative of systemic blood pressure only. As the occlusion cuff was inflated around the large deltoid muscle of the upper arm, it is plausible that cuff inflation to just 60 mmHg around such a muscular area, may have induced a rise in local pressure. These explanations are purely speculative, since investigation of local changes in arterial pressure would require invasive catheterisation in the mid-brachial, which was beyond the scope of this present study. In any event, local pressure changes were not large enough to induce significant alteration in FMD post inflation of the proximal cuff, although it remains a possibility that detrimental retrograde shear effects may have been offset by beneficial transmural pressure impacts.

Despite the speculation that cyclic strain did not play a role in the results of this study, it is possible that the effect of pressure waves did. The physics of haemodynamics is a complex matter which will not be discussed in great length within this chapter. Of note, however, is the interaction between pressure waves and blood flow. It is suggested that reflection of a pressure wave may be associated with an increase in retrograde flow (Heffernan *et al.*, 2013). At a very basic level, the change in pressure between 2 particular points along any vessels length means that transient reversal in flow is accommodated under particular conditions (Nichols *et al.*, 2011). This is based upon the principle that blood flow is dependent on both pressure and downstream resistance. In the present study, the distal cuff induced greater retrograde flow at the site of Doppler measurement than proximal cuff inflation. Certainly, cuff inflation will have affected either pressure or resistance, and possibly both. It could be postulated that pressure waves meeting the

cuffed area were reflected into the path of the oncoming blood flow, acutely increasing retrograde flow above the cuff. In the instance of the proximal cuff, this may have happened more proximally (i.e., in the axillary artery). The change in blood flow/SR pattern in the proximal condition may have been damped by the time it reached the mid brachial artery (site of measurement) and thus would help explain why retrograde SR was not increased as much as in the distal condition. Nevertheless, this explanation does not help to answer the question as to why FMD was not attenuated in the proximal condition despite a significant increase in retrograde SR compared with control.

Another potential explanation pertains to venous congestion. It is widely accepted that cuff inflation to 60 mm Hg occludes venous return to the heart (Wilkinson and Webb, 2001). In the present study, blood would have consequently been congested below the site of cuff inflation. With respect to the distal condition, blood would have been congested in the forearm. In the proximal condition, venous blood would have been congested in the forearm and upper arm, and, more importantly, at the site of Doppler measurement. Venoarterial reflex, also known as postural vasoconstriction reflex, describes the phenomenon of decreased arterial blood flow when a limb is moved from a horizontal to dependent (in this case, vertical) position (Rathbun *et al.*, 2008). This natural reflex is the body's response to an increase in pressure in the venous system, and prevents venous pooling which subsequently prevents loss of fluid into the extravascular space (i.e., oedema). The venoarterial reflex is initiated in any situation where a limb is placed in a dependent position (e.g., lower limbs when sitting). In the present study, venous congestion induced by cuff inflation may have mimicked the action of moving a limb from a non-dependent to dependent position. As such, the body may have

responded by reducing arterial flow and, consequently, SR. It is difficult to interpret whether the differing cuff positions (proximal vs. distal) may have induced differing responses at the site of measurement and the existence of the venarteriolar reflex is still a matter of active debate. Certainly, in the proximal condition, more venous pooling would have occurred at the site of measurement compared to the distal condition. However, since mean SR (Figure 4.2) was similar and diameter the same between conditions, it can be crudely assumed that arterial blood flow was also the same under both conditions (see section 2.4.1). Since SR is the stimulus for arterial adaptation, it seems unlikely that venous pooling, *per se*, was the mediator of opposing FMD responses.

A final feasible elucidation is based around the notion that nitric oxide (NO) production is altered to different degrees of magnitude depending on cuff position. This has previously been investigated in FMD, where it has been observed that FMD with proximal cuff inflation is less NO mediated than distal cuff inflation (Doshi *et al.*, 2001). With this in mind, it may be proposed that prolonged distal cuffing has a bigger impact on FMD decrement than proximal cuffing.

In the individuals where L-FMC could be assessed, a small (yet not significant) constrictor response to low-flow was observed at rest and following cuff inflation in the brachial artery. The topic of whether the brachial artery constricts under conditions of low-flow is widely disputed in the literature (Humphreys *et al.*, 2014). Some studies have found constriction (Levenson *et al.*, 2001; Spiro *et al.*, 2011), whilst others have found paradoxical vasodilation (Thijssen *et al.*, 2008). However, the L-FMC findings herein seem wholly inconsistent and somewhat erratic (see section 6.3.1 for further discussion).

Moreover, the relatively small declines in diameter during low-flow of 0.3-1.5% (i.e., 0.01-0.05 mm for a radial artery) are likely to be associated with significant variation, especially when the spatial resolution of current ultrasound machines is in the range of ~0.01 mm.

In the present study, no significant changes were found in either L-FMC or the composite end point (sum of L-FMC and FMD) under any condition. Although no previous studies have investigated L-FMC under conditions of shear manipulation, the majority of research has reported diminished L-FMC in patients with chronic endothelial dysfunction (Gori *et al.*, 2008; Gori *et al.*, 2010; Harrison *et al.*, 2011; Gori *et al.*, 2012). In terms of acute damage, studies have identified a significantly *augmented* brachial L-FMC following 20 minutes of ischemic-reperfusion injury induced by supersystolic cuff inflation (250-300 mm Hg) (Rakobowchuk *et al.*, 2013; Carter *et al.*, 2014). Moreover, in another study, brachial artery L-FMC was measured in patients with stable and unstable angina (Spiro *et al.*, 2011). Interestingly, those with unstable angina (who had suffered a non-ST segment myocardial infarction) had a greater L-FMC than their healthier counterparts, which is more commonly associated with better endothelial function. These data indicate that brachial L-FMC is currently inconsistent. Certainly it remains to be clarified whether a larger brachial L-FMC is indeed indicative of better endothelial function. More research is necessary to gain better understanding of which factors affect brachial artery L-FMC. Nonetheless, this study suggests that acute exposure to an increase in retrograde shear stress, which has potent detrimental effects on the FMD, does not alter L-FMC.

There are a number of limitations associated with the current study. The sample population is relatively small, but was based on the sample size estimation described

previously. Furthermore, it is greater than many similar studies of this nature (Thijssen *et al.*, 2009b; Tinken *et al.*, 2009; Schreuder *et al.*, 2014). All subjects were young, healthy and free from atherosclerosis. As such, the 30 minute proximal protocol may not have been potent enough to elicit any significant change in FMD, despite a significant increase in retrograde SR. However, since distal cuff inflation did attenuate FMD, it would seem sensible to assume that factors other than SR may have been involved in preservation of FMD in the proximal condition.

Another limitation is that blood flow/SR measurements were taken at rest and then only again after 5 minutes of cuff inflation. It would have been interesting to identify whether cuff inflation to 60 mm Hg instantaneously altered SR pattern. A recent study which also used cuff inflation to attenuate flow/SR demonstrated that cuff inflation around the calf resulted in a change in SR pattern in the superficial femoral artery at the point of the very next blood flow velocity waveform (Heffernan *et al.*, 2013). Although it is likely that the same was true in our study of the upper limbs, further research would be needed to confirm this. It may also be interesting to look at the effect of distal and proximal cuff inflation on pressure waveforms.

Finally, L-FMC was only analysable in 13 of 18 subjects tested. The same sonographer completed all investigations and the same scans were used for both L-FMC and FMD analysis. Therefore, the quality of scanning was not jeopardised in any way between FMD and L-FMC. Seemingly, the technical ability required of a sonographer to complete reliable L-FMC scans is paramount. The L-FMC results obtained within this study are erratic and although the use of L-FMC alongside FMD seems promising, care is warranted when interpreting findings.

4.5 Conclusion

In summary, despite the finding that both proximal and distal cuff positions increase retrograde SR and OSI, vascular function was only attenuated in the distal condition. One may therefore speculate that cuff inflation proximal to the imaged artery (in the ischaemic zone) led to stimuli that counteract the detrimental effect of retrograde shear rate and protect the vessel from the negative effects of retrograde SR during proximal cuff inflation, or that the magnitude of retrograde shear induced by proximal cuff placement fell below the threshold required to impact upon NO-mediated arterial function. Another explanation pertains to the affect of cuff position in itself, given that prolonged distal cuff inflation may have a greater impact upon NO production than proximal cuff inflation. The exact mechanisms are currently unknown, but may relate to local pressure, pressure wave reflection, magnitude of retrograde SR, NO production and/or venous congestion. Changes in L-FMC were small in magnitude and unreliable (see section 6.3.1). Neither proximal nor distal interventions significantly altered L-FMC. Whether L-FMC has any clinical or research use remains enigmatic.

CHAPTER 5 – Study 2
The influence of a venous occlusion plethysmography protocol on arterial shear rate and inflow, and vascular function

5.1 Introduction

The technique of venous occlusion plethysmography (VOP) has been used for over 100 years and is the historical "gold standard" technique used to examine resistance artery blood flow. Forearm VOP involves placement of a strain-gauge around the forearm to measure changes in forearm volume, a surrogate for changes in blood flow. A cuff is placed proximally on the limb and intermittently inflated/deflated to a sub-diastolic pressure. A second cuff is placed around the wrist and inflated to a supra-systolic pressure to exclude the complex hand vasculature from the forearm flow responses. The technique is often combined with local intra-arterial drug administration, which allows for the assessment of changes in blood flow in response to vasoactive substances which are indicative of specific pathways controlling vascular function and health. One limitation of the technique is that no distinction can be made between different vascular beds within the forearm (e.g., micro/macro-circulation, skin/muscle). Another important assumption is that only venous return is occluded during VOP, and arterial blood flow remains unaffected by cuff inflation.

In 1989, a research group sought to question the assumption that VOP selectively blocks venous outflow, without impacting upon the arterial inflow. They designed an array of small studies. In one study of 5 subjects, a proximal cuff was inflated around the upper arm for 30 seconds to 20, 40 and 60 mmHg. However, no wrist cuff was used. Blood flow velocity was measured in the radial artery using Doppler ultrasound. The results identified an inverse dose-response of blood flow velocity to cuff pressure (i.e., velocity was decreased the most at 60 mmHg). Despite several methodological shortcomings, the

results clearly suggest that even a short burst of subdiastolic cuff inflation significantly alters arterial inflow.

The primary purpose of this present pilot study is to definitively explore whether the widely adopted VOP protocol alters shear rate (SR) pattern, and thus flow, in the brachial artery in healthy volunteers. Based on earlier observations in study 1, the hypothesis for this pilot study is that forearm VOP will attenuate flow in the brachial artery during the periods of cuff inflation, largely through an increase in retrograde SR. Furthermore, it is believed that repeated exposure to elevations in retrograde SR may impair endothelial function. Therefore, the secondary objective is to assess whether a standardised VOP protocol, as typically used in studies using multiple doses of drugs to examine endothelial function, impacts conduit artery endothelium dependent nitric oxide-mediated flow mediated dilation.

5.2 Methods

5.2.1 Sample size estimation

The effect of venous occlusion plethysmography on shear rate pattern has not been previously investigated. Therefore, sample size estimation was not performed prior to commencing this pilot study.

5.2.2 Participants

Eight young healthy males (age 26.0 ± 2.3 yr, height 1.80 ± 0.07 m, weight 77.67 ± 10.43 kg, BMI 23.89 ± 2.45 kg/m^2) were recruited and screened for this pilot study. Exclusion criteria were the same as study 1. The study procedures were approved by the ethics

committee of Liverpool John Moores University and adhered to the Declaration of Helsinki. Informed written consent was obtained from all participants prior to experimental testing.

5.2.3 Experimental design

Participants attended the laboratory on one occasion. Flow mediated dilation was assessed in the right brachial artery before and after 4 x 5 minute cycles of venous occlusion plethysmography (VOP). The VOP cycle consisted of repeated periods of 10 seconds inflation of the blood pressure cuff to 50 mmHg, which was placed proximally around the upper arm, followed by 10 s of deflation. Each 5-minute VOP cycle was interspaced by a 5 minute passive rest period resulting in a 40 minute intervention period (Figure 5.1).

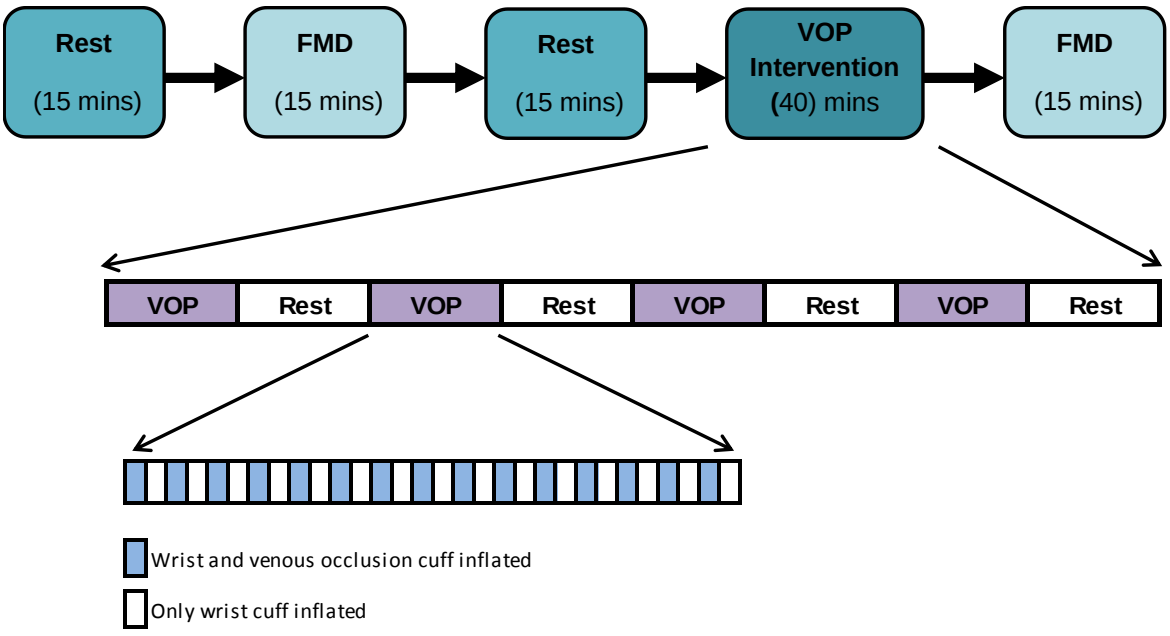


Figure 5.1. Study 2 experimental design

5.2.4 Experimental procedures

On the testing day, height and weight were measured as described previously and BMI calculated. Subjects rested in a supine position for ≥15 minutes to reach physiological homeostasis. Heart rate, SBP and DBP were recorded from an automated sphygmomanometer placed around the upper left arm. Following these measurements, FMD was assessed. Following 15 minutes of supine rest after the initial FMD to allow brachial artery diameter and blood flow to return to homeostasis, baseline diameter and blood flow velocity were recorded as pre-intervention baseline values. Subsequently, subjects underwent the intervention, which consisted of 5 minutes of VOP followed by 5 minutes of passive rest, repeated four times. The VOP phase involved inflation of an automatic pneumatic cuff (SC-5 cuff, E-20 rapid cuff inflator; D.E Hokanson, WA, USA) around the wrist to 220 mm Hg for the entire 5 minute duration. An additional cuff was placed around the upper third of the upper arm that was intermittently inflated/deflated in 10 second intervals to 50 mm Hg. This protocol is typically adopted in studies that use VOP in combination with intra-arterial infusion of vasoactive substances in varying doses. At the end of the VOP cycle, both cuffs were deflated simultaneously, immediately prior to commencing the 5 minute rest phase (Figure 5.1). Artery diameter and velocity were recorded continuously throughout each 5 minute VOP cycle and for the first 3 minutes of each rest period. The ultrasound probe remained in the same position throughout the entire 40 minute intervention. After the final VOP cycle, blood pressure and heart rate were again recorded. Immediately following the 40 minute intervention, FMD was repeated. All testing sessions were completed by the same sonographer. In a subgroup of 4 participants, an additional 30 second concurrent mean blood flow velocity and diameter

recording was completed with only the wrist cuff inflated, immediately prior to commencing the VOP protocol.

5.2.5 Data analysis

Full details of all data analysis for FMD, L-FMC, composite end point, artery diameter, shear rate and oscillatory shear index can be found in chapter 3.

5.2.5.i Change in diameter induced by VOP

Data recorded from the rest phases of the VOP protocol (study 2) were analysed using the same software as before. To identify whether VOP caused a change in vessel diameter, data were analysed in a similar way to FMD. Diameter during the last 30 seconds of each rest phase recording (end diameter) was used as equivalent baseline. This decision was based on the assumption that after 3 minutes, diameter should have returned to baseline levels, as is true of FMD. As with the FMD analysis above, outputs of end diameter (mm), peak diameter (mm), relative change in diameter (% change from end diameter), TTP (s) and SR_{AUC} were calculated automatically.

5.2.6 Statistical analysis

5.2.6.i Blood pressure, heart rate, shear rate, blood flow, diameter and flow mediated dilation

Changes in SBP, DBP, HR, shear rate, blood flow, diameter and FMD data were analysed using one-factor (time) LMMs. Flow mediated dilation data were adjusted to account for baseline diameter and SR_{AUC} as per study 1.

5.2.6.ii Artery dilation following each venous occlusion cycle

To investigate whether each VOP cycle induced dilation during the rest phase, a two-factor LMM (4 rest phase x 2 diameter [end vs. peak diameter]) was performed.

5.3 Results

5.3.1 Effect of VOP on blood pressure, blood flow, shear rate and oscillatory shear index

Baseline SBP, DBP, HR shear rate (SR) and OSI are presented in tables 5.1 and 5.2. There were no significant changes in DBP or SBP over time (Table 5.1). Heart rate significantly dropped from baseline (61 ± 20 vs. 54 ± 20 bpm; *P*=0.036).

Table 5.1. Blood pressure and heart rate before and after 4th VOP cycle (n=8)

Parameter	Baseline	After 4th VOP cycle	LMM *P*
SBP, mm Hg	118 ± 11	116 ± 11	0.582
DBP, mm Hg	65 ± 5	68 ± 5	0.077
Heart rate, bpm	61 ± 20	54 ± 20	0.036

P value refers to one-factor LMM (time) main effect. A significant main effect was detected in HR.
DBP, diastolic blood pressure; **HR**, heart rate; **MAP**, mean arterial pressure; **SBP**, systolic blood pressure

To investigate the effect of each venous occlusion plethysmography (VOP) cycle on shear pattern, the average of mean, antegrade and retrograde SR at baseline and during each 5 minute cycle were compared. Mean and antegrade SR were significantly lower during each of the four VOP cycles compared to baseline (*P*<0.0005). Retrograde SR was significantly increased in all four VOP cycles compared to baseline (*P*≤0.005; Table 5.2). There were no significant differences in SR pattern between VOP cycles. Oscillatory shear index was also significantly increased in all four VOP cycles compared with baseline (*P*<0.0005). Changes in blood flow were also investigated. Blood flow was significantly decreased from baseline during all four VOP cycles (*P*<0.0005). Again, there were no significant differences in any parameter between VOP cycles (Table 5.2). In a small subgroup of four participants, blood flow velocity and diameter were recorded with only

the wrist cuff inflated. Wrist cuff inflation (220 mm Hg) significantly increased retrograde SR and decreased mean and antegrade SR ($P \leq 0.019$). Additionally, blood flow was significantly decreased from baseline with wrist cuff inflation ($P=0.010$). In the same four subjects, SR pattern and blood flow did not significantly change from wrist cuff inflation only, to VOP (wrist and venous occlusion cuff inflation) ($P \geq 0.357$).

Table 5.2. Shear rate and blood flow characteristics of healthy subjects before and during each VOP cycle (n=8)

Parameter	Baseline	Cycle 1	Cycle 2	Cycle 3	Cycle 4	LMM *P*
Mean SR, s^{-1}	85 ± 53	30 ± 55*	27 ± 53*	34 ± 53*	31 ± 53*	<0.0005
Antegrade SR, s^{-1}	97 ± 49	54 ± 50*	54 ± 49*	55 ± 49*	54 ± 49*	<0.0005
Retrograde SR, s^{-1}	-12 ± 20	-24 ± 20*	-26 ± 20*	-20 ± 20*	-22 ± 20*	0.001
OSI, a.u	0.14 ± 0.16	0.31 ± 0.16*	0.32 ± 0.16*	0.28 ± 0.16*	0.30 ± 0.16*	<0.0005
Blood flow, $ml \cdot min^{-1}$	505 ± 204	184 ± 211*	161 ± 204*	200 ± 204*	181 ± 204*	<0.0005
BF velocity ($cm \cdot s^{-1}$)	8.4 ± 3.4	3.0 ± 3.5*	2.7 ± 3.4*	3.3 ± 3.4*	3.0 ± 3.4*	<0.0005

P value refers to one-factor LMM (time) main effect. A significant main effect of time was found in all parameters. Pairwise comparisons: *significantly different from baseline
a.u, arbitrary units; **OSI**, oscillatory shear index; **SR**, shear rate; **VOP**, venous occlusion plethysmography

Table 5.3. Shear rate and blood flow at baseline, with wrist cuff inflation only and during VOP (n=4)

Parameter	Baseline	Wrist Cuff Only	Cycles 1-4	LMM *P*
Mean SR, s^{-1}	127 ± 91	39 ± 91*	46 ± 91*	0.020
Antegrade SR, s^{-1}	133 ± 86	66 ± 86*	68 ± 86*	0.032
Retrograde SR, s^{-1}	-6 ± 13	-37 ± 13*	-22 ± 13*	0.011
OSI, a.u	0.07 ± 0.16	0.30 ± .16*	0.25 ± 0.16*	0.003
Blood flow, $ml \cdot min^{-1}$	748 ± 242	231 ± 242	269 ± 242	0.017
BF velocity ($cm \cdot s^{-1}$)	12.5 ± 4.0	3.9 ± 4.0*	4.5 ± 4.0*	0.017

P value refers to one-factor LMM (time) main effect. A significant main effect of time was detected in all parameters. Pairwise comparisons: *significantly different from baseline. **a.u**, arbitrary units; **OSI**, oscillatory shear index; **SR**, shear rate; **VOP**, venous occlusion plethysmography

5.3.2 Effect of each VOP cycle on diameter

Following each 5 minute VOP cycle, the wrist cuff and occlusion cuffs were simultaneously deflated before commencing the 5 minute rest period. After each cycle, a significant increase in diameter was observed in response to relative reactive hyperaemia. The magnitude of change in diameter did not change across the 4 VOP cycles. In addition, diameter and blood flow returned to baseline levels at the end of each 5-minute resting period. Following detection of a significant main effect of time (i.e., peak diameter vs. end diameter), pairwise comparisons confirmed peak diameter was significantly greater than the diameter during the last 30 seconds of recording, in all four rest periods ($P \leq 0.008$; Table 5.4; Figure 5.2). Additionally, diameter changes, SR stimulus (as measured by SR_{AUC}) and time to peak (TTP) were compared between all four cycles using a one-factor LMM (Table. 5.4). There were no significant differences in any parameter between cycles.

Table 5.4. Change in diameter induced by reactive hyperaemia following each VOP cycle (n=8)

Parameter	Rest 1	Rest 2	Rest 3	Rest 4	LMM *P*
End D, mm	3.90 ± 0.06	3.91 ± 0.06	3.87 ± 0.06	3.85 ± 0.06	0.541
Peak D, mm	4.05 ± 0.06*	4.05 ± 0.06*	4.02 ± 0.06*	3.97 ± 0.06*	0.387
Change in D, %	3.92 ± 2.70	3.66 ± 2.70	3.88 ± 2.70	3.14 ± 2.70	0.422
TTP, s^{-1}	50.2 ± 27.4	38.3 ± 27.4	36.7 ± 27.4	43.9 ± 27.4	0.270
SR_{AUC}, a.u 10^4	10.8 ± 10.3	10.7 ± 10.3	8.7 ± 10.3	10.8 ± 10.3	0.387

P value refers to one-factor LMM (time) main effect. No significant main effects of time were detected.
Two-factor LMM pairwise comparisons: *significantly dilated compared with end diameter $P \leq 0.008$.
a.u, arbitrary units; **SR_{AUC},** shear rate area under the curve; **D**, diameter; **TTP**, time to peak; **VOP**, venous occlusion plethysmography

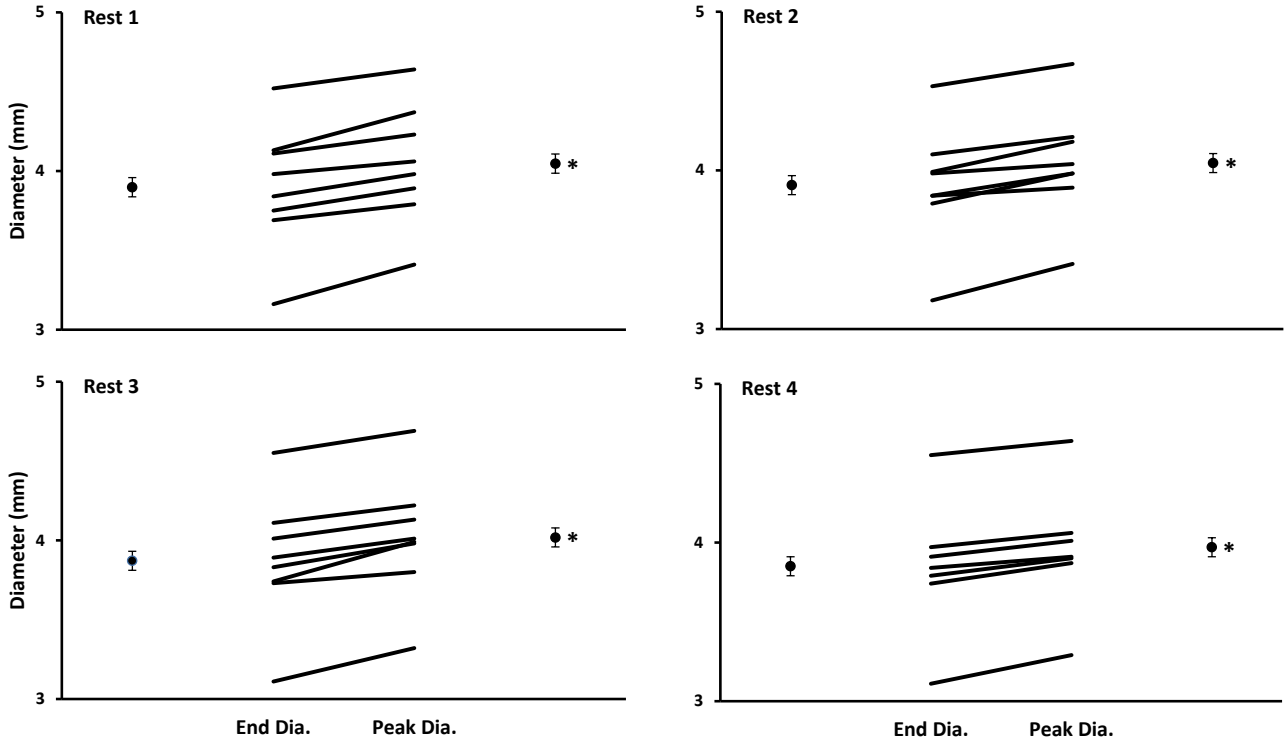


Figure 5.2. Artery diameter during the last 30 seconds of recording and at peak during rest phase
Pairwise comparisons: *significantly different from end diameter ($P \leq 0.008$). Error bars represent SD.

5.3.3 Effect of each VOP cycle on flow mediated dilation

Flow mediated dilation was assessed before and after the 40 minute VOP intervention. There were no significant differences between pre- and post- measurements in any parameter except SR_{AUC} which was significantly reduced after the VOP protocol (29.4 ± 17.4 vs. 23.6 ± 17.4; *P*=0.048). In addition to analysis of FMD% with covariate control (as presented in Table 5.5), FMD% was analysed without adjustment for baseline and $SR_{AUC.}$ Again, no significant difference was detected.

Table 5.5. Brachial flow mediated dilation of healthy subjects before and after 4 VOP cycles (n=8)

Parameter	Before	After	LMM *P*
Baseline D, mm	3.88 ± 0.06	3.79 ± 0.06	0.089
Peak D, mm	4.18 ± 0.05	4.07 ± 0.05	0.038
FMD, %	7.83 ± 5.65	7.48 ± 5.65	0.731
Adj. FMD, %	7.68 ± 4.63	7.47 ± 4.63	0.837
TTP, s^{-1}	54.2 ± 25.9	53.5 ± 25.9	0.934
SR_{AUC}, a.u 10^4	29.4 ± 17.4	23.6 ± 17.4*	0.048

P value refers to one-factor LMM (time) main effect. No significant interactions were detected.
Adj, FMD, adjusted flow mediated dilation; **a.u**, arbitrary units; **SR_{AUC},** shear rate area under the curve; **D**, diameter; **FMD**, flow mediated dilation; **TTP**, time to peak.

5.4 Discussion

The primary objective of this present study was to explore whether a forearm venous occlusion plethysmography (VOP) protocol would induce local changes in arterial shear rate (SR) pattern. The secondary aim was to identify whether VOP, by means of repeated changes to the SR pattern, has potential detrimental effects on vascular function by assessing FMD before and after a typical VOP protocol. This is the first study to investigate the effect of VOP on SR pattern, blood flow and vascular function. The principle findings were that forearm VOP significantly altered SR pattern by increasing retrograde SR and decreasing mean SR. In a small subset of participants, it was demonstrated that this wrist cuff inflation to 220 mm Hg alone, also caused a significant alteration to SR pattern. Secondary analysis identified that the VOP protocol induced reactive hyperaemia as evidenced by a small, but significant dilation during the rest phases which immediately followed each 5 minute VOP cycle. However, diameter and shear rate normalised within the 5-minute resting period.

The results from the present study challenge, for the first time, the assumption that arterial inflow is unchanged during the classical VOP procedure. The findings clearly highlight that arterial inflow (ml·min) was significantly reduced by more than 50% during the VOP protocol. Whilst there were large changes in mean, antegrade and retrograde SR, the most apparent attenuation was noted in antegrade SR, which was halved during all four VOP cycles. Similar studies involving continuous distal cuff inflation (around the forearm) to a low pressure (30-75 mm Hg) have demonstrated no change in brachial antegrade SR (Thijssen *et al.*, 2009b) or a paradoxical increase in brachial antegrade SR (Schreuder *et al.*, 2014). In these studies however, the cuff was in close proximity to the

site of measurement. In this present study, the distal cuff was inflated around the wrist, with measurements made in the brachial artery. Despite the wrist cuff being at a greater distance from the site of measurement, inflation of the wrist cuff to 220 mm Hg greatly attenuated antegrade SR in the brachial artery. This is likely to be due to a large increase in downstream resistance, combined with a negligible (if any) increase in local pressure, thereby decreasing antegrade SR.

Although the VOP protocol significantly altered SR pattern, there was no significant change in FMD from pre to post intervention. This observation somewhat contrasts with previous findings. In these studies, an acute detrimental effect of retrograde SR on FMD in the brachial and femoral arteries has been highlighted (Thijssen *et al.*, 2009b; Tinken *et al.*, 2009; Johnson *et al.*, 2012; Schreuder *et al.*, 2014). There may be several explanations for the disparate results. Firstly, the protocols used were different. In the previous studies, cuff inflation was continuous, while in the present study, an intermittent protocol was used. Indeed, during the 5 minutes of rest, reactive hyperaemia was observed, that induced a significant dilatory response, which may have protected the vessel from the acute effects of retrograde SR. Finally, the site of measurement of SR and FMD was between the wrist cuff and upper arm occlusion cuff. The results in study 1 identified that proximal and distal cuffs have opposing effects on vascular function. It may be the case that in this study, these effects counteracted each other. However, since the wrist cuff was inflated continuously for each 5 minute protocol to 220 mm Hg, it would seem more likely that it would have had an overriding negative effect on vascular function. Nevertheless, FMD was not reduced following four bouts of VOP, suggesting that the rest period between the bouts served an important role in preservation of vascular function.

An alternative explanation pertains to the magnitude of retrograde SR. Although retrograde SR was significantly increased during the VOP protocol, this may not have been enough to elicit a detrimental effect on FMD. Certainly, a dose-response of increase in retrograde SR and decline in FMD has been observed (Thijssen *et al.*, 2009b). The results herein give rise to the suggestion that there is a retrograde SR 'threshold' whereby endothelial function is only attenuated when retrograde SR reaches a certain level. Under some conditions, for example exercise, however, the concurrent increase in antegrade SR would likely negate any negative effects of the retrograde SR (Newcomer *et al.*, 2011).

The fact that VOP was found not to be detrimental to vascular function is note-worthy. VOP is frequently used in studies examining resistance artery vascular tone and endothelial function. If VOP had caused a significant change in FMD, fundamental questions could have been asked about its ability to reliably assess vascular function. In the present study, changes in arterial inflow/SR were uniform across all four VOP cycles which would lead one to believe that any change in arterial flow/SR is consistent. Since FMD was not significantly altered following four repeated bouts of VOP, the finding that SR pattern was altered, cannot explain the disparity when comparing and correlating different techniques of measuring vascular function. One previous study investigating the effects of exercise training on nitric oxide-mediated resistance artery function (using VOP with drug infusion) and conduit artery function (using FMD), found no relationship in the magnitude of improvement in the two techniques (Green *et al.*, 2004b). Others have also found similar disparate findings between VOP and FMD (Celermajer *et al.*, 1994; Taddei *et al.*, 1995). In another study specifically investigating the relationship between the two techniques in young and older men, it was concluded that forearm VOP (with ACh

infusion) and FMD do not provide the same information on peripheral vascular function, and cannot be used as surrogate measures for each other(Eskurza *et al.*, 2001). One reason for the differing results pertained to heterogeneity between the vascular beds. Venous occlusion plethysmography measures the vascular response of the arterioles whilst FMD records the response of the conduit arteries. Previous work has highlighted that production of NO is greatest in the larger arteries (Griffith *et al.*, 1987), which may explain why the two measures cannot be used as surrogates of each other.

There are several limitations to the present pilot study. Firstly, the sample size was small, however, it has given clear rationale for further research in this area. Investigation into whether wrist cuff alone, or wrist and venous occlusion cuff inflation is the key mediator to changes in SR pattern, is paramount. However, this was only tested in a subset of four participants. Whilst there is some evidence that the wrist cuff caused the majority of change in SR pattern, the sample size was too small to make firm inferences. Furthermore, the study did not examine SR pattern with only the intermittent cuff inflated. Future studies may also look to examine whether the intermittent venous occlusion cuff alone had any effect upon SR pattern. One final limitation pertains to the anatomy of the arm. Although the brachial artery is the main conduit vessel of the upper arm, there are other arterial pathways including the profunda brachii artery. Moreover, it is common to have a bifid brachial system where the brachial artery bifurcates in the proximal upper arm. As flow/SR was only measured in the brachial artery only, it is unknown whether blood was redistributed to other arterial vessels. In a true VOP study, redistribution would not matter, as blood volume/inflow measurements are based on the whole section of limb, and not a particular vessel.

5.5 Conclusion

In summary, the results from the study have provided clear evidence that arterial SR and arterial inflow in the brachial artery is consistently altered during a protocol of repeated VOP. This provides evidence that the important assumption of VOP (i.e., cuff inflation to subdiastolic levels only influences venous outflow) is not met. Nonetheless, this does not alter brachial artery endothelial function. As such, error induced by arterial inflow alteration is likely to be consistent both within and between subjects.

CHAPTER 6 - Synthesis

6.1 Overview

The research described in the present thesis was designed to investigate the effects of shear rate (SR) manipulation on vascular function in healthy humans. Previous studies have highlighted that cuff inflation distal to the site of measurement augments retrograde flow which is subsequently detrimental to acute vascular function. However, cuff inflation performed with the cuff in other orientations has not been investigated. Doppler ultrasound was used to measure SR pattern in the brachial artery under various perturbations. Study 1 focussed on examining the acute effects of SR manipulation on SR pattern, and subsequently, nitric oxide (NO)-mediated endothelium dependent vascular function (FMD) and low-flow mediated constriction (L-FMC). Results from study 1 informed study 2, where a pilot study was implemented to explore whether the established technique of forearm venous occlusion plethysmography (VOP) altered arterial inflow/SR and, furthermore, if these changes impacted upon brachial artery FMD.

6.2 Major findings

The main novel finding from study 1 was that proximal cuff inflation induced a significant change in shear rate (SR) pattern in the brachial artery below the occlusion cuff. However, this change in SR did not impact vascular function, as indexed by FMD. This finding was not as expected, since retrograde SR was significantly increased from baseline. Indeed, previous data suggests that the magnitude of retrograde SR observed in the proximal condition should have been enough to detrimentally impact upon FMD (Thijssen *et al.*, 2009b). Furthermore, a similar oscillatory shear index (OSI) between distal and proximal conditions further supports the suggestion that proximal cuff inflation should have had a

negative effect on FMD since animal and human research indicates that oscillatory shear, characterised by increased retrograde and decreased mean SR, elicits a pro-atherogenic phenotype (Chappell *et al.*, 1998; Ziegler *et al.*, 1998; Silacci *et al.*, 2001). This can be detrimental to vascular function even in acute bouts. However, in the present study, despite similar OSI between distal and proximal conditions, changes in vascular function were not the same between experimental conditions. Therefore, it is possible that factors other than SR may have influenced FMD following proximal cuff inflation including, changes in local pressure, pressure wave reflection and/or venous congestion. Additionally, it could be argued that the increase in retrograde SR was not large enough to elicit a change in FMD.

In study 2, the main novel finding was that forearm VOP involving wrist cuff inflation to 220 mm Hg and intermittent upper arm cuff occlusion to 50 mm Hg, significantly altered SR pattern and decreased blood flow in the brachial artery. In a subgroup, it was highlighted that the wrist cuff alone induced similar changes to SR pattern and blood flow. Vascular function was also assessed before and after four VOP cycles, with no significant difference evident from pre to post procedure, despite the marked changes in shear rate pattern induced by the (dis)continuous inflation of blood pressure cuffs around the arm.

6.3 General discussion

6.3.1 Synthesis of study 1 and 2

In study 1, a detrimental effect of 30 minutes of continuous distal cuff inflation to 60 mm Hg was demonstrated. However, there was no detrimental effect of proximal cuff inflation. In study 2, despite large changes in SR pattern induced by the VOP protocol, there was, again, no detrimental effect on FMD. The most pertinent question that has arisen from these major findings pertains to why there was no change in FMD after proximal cuff inflation or VOP, despite increases in antegrade SR.

With regard to the proximal condition in study 1, the position of the cuff may have protected the downstream vessel from the negative effects of increased retrograde SR. Due to the study design and lack of invasive measures, the underlying mechanisms responsible for these findings remain unknown. However, it could be postulated that several factors may have influenced results, including an increase in local arterial pressure, the effect of cuff position on the pressure waveform or an effect of venous congestion.

Cuff inflation during the VOP protocol was intermittent in nature with the wrist cuff inflated for a total of only 20 minutes during the entire 40 minute VOP protocol and the upper occlusion cuff only inflated for 10 minutes of the protocol. One explanation for the lack of change in FMD may be that the VOP stimulus was not long enough in duration to elicit any change. Indeed, to reliably compare with the 30 minutes of continuous inflation, it may have been better to perform a 60 minute protocol. Nevertheless, it would be unusual for any VOP protocol to last longer than 40 minutes, in which case, findings from a longer protocol would not be applicable to practice. Another plausible explanation is

that each VOP cycle with wrist inflation to 220 mm Hg acted in the same way as ischaemic pre-condition (IPC). Remote IPC has been shown to protect against acute endothelial damage (Bailey *et al.*, 2012) and typically involves 40 minutes of intermittent cuff inflation/deflation (5:5 minutes; 220 mm Hg) around an area of large muscle mass. In the present VOP study, a small, yet significant dilator response in all 8 subjects was detected during each of the rest phases, indicating that repeated reactive hyperaemia was induced by the inflation of both cuffs. It may in turn be possible that this in some way protected the artery, in a similar way to IPC. In the study cited above, the acute detrimental effect of strenuous exercise on FMD was abolished when the exercise bout was preceded by IPC, highlighting it as a potent stimulus for preservation of vascular function (Bailey *et al.*, 2012). It is possible that the 5 minute break between each bout of VOP (where reactive hyperaemia and vasodilation were apparent), may have counteracted any negative effects of increased retrograde SR during the VOP protocol itself. Put simply, there may be countervailing impacts on endothelial function of the brachial artery of retrograde flow induced by wrist cuff inflation (negative impact) and repeated wrist cuff deflation (IPC-mediated positive impact), resulting in an overall lack of change in FMD

Another novel aspect of study 1 was the use of low-flow mediated constriction (L-FMC) as an additional method of evaluating vascular function. One important factor, alluded to previously in chapter 3, is the issue of variation in L-FMC. For exploratory purposes, subsequent calculation of coefficients of variation (CVs) for adjusted FMD% and adjusted L-FMC% (n=13) were performed. The within-day CVs were calculated from the pre and post control data. The within-day CV for FMD of 12% is in keeping with previous research (Herrington *et al.*, 2001; Charakida *et al.*, 2013). On the contrary, the CV for L-FMC of 134%

is worryingly large. Whilst it is unusual to be presented with CVs over 100%, it is not impossible and relates to the fact that the standard deviation is much greater than the mean value. Added to this, the direction of the L-FMC response both within and between trials was not uniform. For example, most participants constricted in response to low-flow, but some paradoxically dilated. This will have greatly affected the CV. The scatter plots of within-day FMD and L-FMC (Figure 6.1) help to illustrate the erratic nature of the L-FMC data. Although a statistically unsound method (since data are repeated measures using the same subjects), the r^2 values (FMD, 0.797; L-FMC, 0.022) again highlight how unreliable the L-FMC data is. Furthermore, all L-FMC results were taken from the same scans as the FMD data, which were performed by the same sonographer throughout. Even with this internal control, several scans were not suitable for analysis. Taken together, these factors highlight that L-FMC potentially has high systematic error and caution is required when collecting and interpreting data.

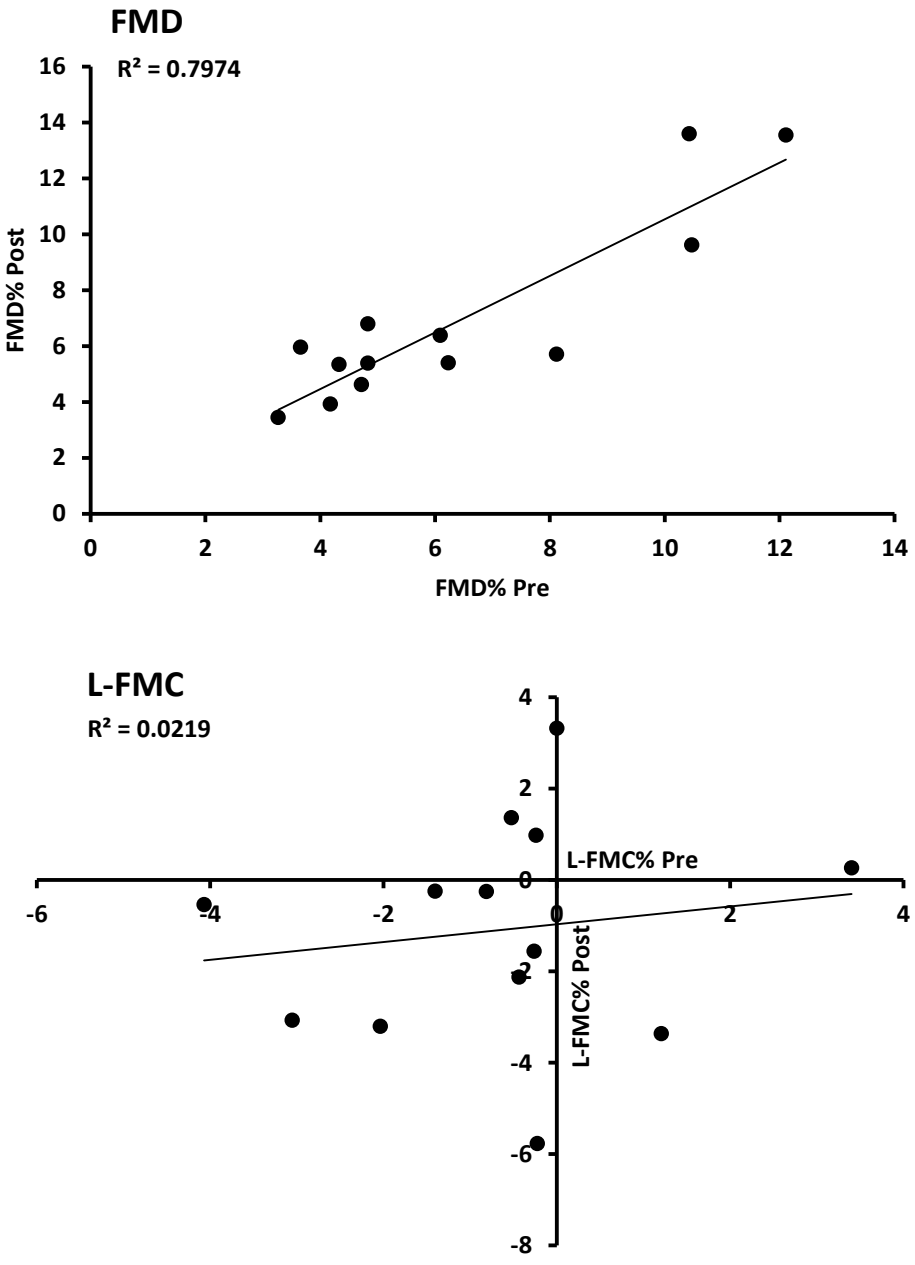


Figure 6.1. Graphical representation of within-day FMD and L-FMC values
FMD; Flow mediated dilation; **L-FMC;** Low-flow mediated constriction

6.3.2 Clinical implications of the findings

One may look favourably on some of the findings and their extrapolation to a clinical setting. In the instance of the VOP protocol, the intermittent protocol caused an unexpected yet potentially protective reperfusion and dilator response, akin to remote ischaemic pre-conditioning (IPC). With more research, intermittent cuff inflation at a low sub-diastolic pressure may have some benefit to patients whose cardiovascular presentation would contraindicate IPC cuff inflation to typically high pressures >200 mmHg.

With regard to shear rate, some diseases / conditions induce retrograde flow in conduit and resistance vessels. For instance, the coronary arteries in aortic valve disease (Carroll and Falsetti, 1976) and the vertebral arteries in subclavian steal syndrome (Hennerici *et al.*, 1988) present with retrograde flow during some or all of the cardiac cycle. Since even acute doses of retrograde flow have been shown as detrimental to vascular function (Thijssen *et al.*, 2009b; Tinken *et al.*, 2009), prolonged exposure of these vessels to retrograde flow may impact upon vascular health. Future work could look to address whether vascular function is attenuated in these affected vessels.

On the contrary, some of the techniques used within this thesis would not be appropriate for use in certain clinical populations. The vasculature is a complex system which is prone to development of atherosclerosis, leading to severe stenosis, acute thrombosis and haemorrhage. Although on a slight tangent from the findings of this thesis, it is important to stress that conducting cuff inflation based studies in some clinical populations may be unsafe. For example, in patients who are predisposed to deep vein thrombosis (DVT), cuff inflation may also cause a risk. Virchow's triad of coagulability describes the three major factors of increase risk of DVT – hypercoagulability, blood stasis and endothelial injury (Lowe, 2003). Since it could be argued that the studies herein induced venous blood stasis and endothelial injury, the risk of DVT to susceptible patients is a real

threat. Cuff inflation studies / interventions may also not be suitable for patients with severe peripheral artery stenosis as any increase in pressure could induce critical limb ischemia.

6.3.3 Methodological consideration and limitations

There are many methodological considerations and limitations to the work presented in this thesis that will be briefly discussed.

Study 1 was conducted as a within-subjects trial which is beneficial as participants act as their own control. It also aids identification of cause and effect as baseline variables were similar between conditions. All testing was conducted at the same time of day to avoid circadian variation in blood pressure, heart rate and other physiological variables. Moreover, FMD and L-FMC were conducted in line with current guidelines (Gori *et al.*, 2008; Thijssen *et al.*, 2011a). Study 2 was conducted as a pilot study and as such, there was no control data. Future studies should look to implement a within-subjects controlled study design.

There were also several limitations to both studies presented herein. In study 1, it was suggested that changes in local pressure or pressure waves may have been the reason for no change in FMD in the proximal condition. However, this is purely speculative as the study was non-invasive and local pressures were not recorded. Moreover, no pressure wave analysis was completed. Although the distal cuff induced attenuation of brachial FMD at 60 mmHg, there is potential that this was not potent enough in the proximal condition. Future research should adopt a greater subdiastolic cuff pressure (e.g., 10 mm Hg lower than each individuals diastolic BP) Study 2 has many limitations. As mentioned

previously, there was no control group, and as such, cause and effect cannot be proven. Furthermore, the sample size of 8 is extremely small and therefore the study was potentially underpowered. Future studies should use the results herein to perform a formal sample estimation calculation. Finally, SR measurement with only the wrist cuff inflated was completed in 4 subjects. This sample size is not large enough to make meaningful inferences. Despite this, it was interesting to note that the wrist cuff inflation to 220 mm Hg did induce a significant decrease in mean SR and increase in retrograde SR, even with such a small sample size. Further research should incorporate recording changes in SR with a) wrist cuff only inflated; b) intermittent cuff inflated only and c) both cuffs inflated. This would help to deduce the precise effects of both cuffs individually, and together, on SR pattern. Furthermore, it would be interesting to investigate changes in local pressure using invasive catheterisation.

6.4 Directions for future research

The present thesis has identified that proximal cuff inflation does not affect vascular function despite increased retrograde SR. It has also demonstrated that a forearm VOP protocol also does not alter FMD, again, despite increased retrograde SR. This is in contrast to distal cuff inflation which attenuated FMD.

There are many questions that have arisen from these findings that warrant future research. Firstly, with regard to study 1, it would be interesting to know whether distal and proximal cuff inflation induce variant pressure waves. If different pressure waves are

identified, this could help to deduce why FMD is not affected by proximal cuff inflation. Additionally, invasive local blood pressure measurement using arterial catheterisation could also be investigated. However, careful methodological considerations would be needed in order to achieve cuff inflation for 20-30 minutes and concurrent invasive blood pressure measurement in a manner that is safe for the participant.

In addition to the effect of sub-diastolic cuff inflation, work has previously investigated the effect of a proximal versus distal cuff placement when performing FMD. Interestingly, a recent meta-analysis reported that FMD performed with a proximal cuff is at least as prognostic as when using a distal cuff (Green *et al.*, 2011). More importantly, this was true despite the observation that FMD using a proximal cuff is less reliant on NO-mediated vasodilation than when using a distal cuff (Doshi *et al.*, 2001). Although difficult to compare these findings to those from study 1, it does suggest that proximal and distal cuffs perhaps offer different stimuli to each other. In terms of research practice, this may have implications in studies where FMD is undertaken multiple times over a few hours, in the same subject. If repeated FMD using a distal cuff was found to have a detrimental effect on vascular function, then perhaps using a proximal cuff would produce more reliable and clinically relevant results.

With regard to L-FMC, the literature has identified it as a tool that could enhance the prognostic capacity of FMD when the two measures are used concurrently(Humphreys *et al.*, 2014). However, the results in the present thesis have highlighted that L-FMC (in the brachial artery at least) is far from a reliable technique. Certainly, previous results have

been more uniform in the radial artery (Gori *et al.*, 2008; Gori *et al.*, 2010; Dawson *et al.*, 2012; Gori *et al.*, 2012). Nevertheless, since the majority of FMD research is conducted using the brachial artery as the conduit of choice, it would seem sensible to further examine whether there is true heterogeneity between radial and brachial L-FMC. Furthermore, it is vital to explore the clinical meaningfulness of the assessment of L-FMC and the composite end point.

With regard to VOP, future research might seek to fully answer the question as to whether arterial inflow truly is altered. The results herein, infer that this is the case, but the sample size was small. The use of VOP as a gold standard measure of vascular function is likely to continue for many years to come. It may also be interesting to investigate whether there is any beneficial effect of a VOP protocol on vascular function. If this were true, then similar protocols could perhaps be used in a therapeutic capacity.

To conclude, this thesis has investigated the effects of various methods of altering SR pattern using cuff inflation, on traditional and novel indices of vascular function. The area of haemodynamic pathophysiology is complex and whilst the findings have answered some of the original research questions, understanding of the mechanisms is far from complete. Further research is certainly required to further understand the mechanisms involved and to answer new questions that have now arisen.

CHAPTER 7 – References

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