Brachial Artery Flow-Mediated Dilation in Humans: Establishing Age- and Sex-Related Reference Values with a Focus on the Role of Shear Stress

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List of Abbreviations

ANOVA	Analysis of variance
ATP-III	Adult Treatment Panel III
BMI	Body mass index
CVD	Cardiovascular disease
EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
FMD	Flow-mediated dilation
GTN	Glyceryl trinitrate
HDL	High-density lipoprotein
HRT	Hormone replacement therapy
IMT	Intima-media thickness
LDL	Low-density lipoprotein
MetS	Metabolic syndrome
NO	Nitric oxide
ORα	Oestrogen receptor alpha
ORβ	Oestrogen receptor beta
SD	Standard deviation
SRAUC	Shear rate area-under-the-curve

Abstract

Cardiovascular disease (CVD) is the world-leading cause of mortality. The pathophysiological process underlying CVD is atherosclerosis, often preceded by dysfunction of the endothelium, the inner layer of the artery wall. The endothelium is sensitive to hemodynamic stimuli, including shear stress (i.e. friction force of flowing blood). Endothelial function, assessed using brachial artery flow-mediated dilation (FMD), is a predictor of future CVD risk. In brief, the FMD test consists of ultrasonic assessment of the relative change in brachial artery diameter before and after a 5-minute period of distal limb (forearm) ischaemia, induced by cuff occlusion. Releasing the cuff leads to a transient increase in shear stress through the brachial artery (i.e. shear rate area-under-the-curve; SRAUC), which in turn stimulates vasodilation. Since the introduction of the FMD technique in 1992, efforts have been made to standardise the methodology for the performance of the FMD and expert-consensus protocol guidelines were established. However, large variability in FMD data is present within the literature, likely due to poor adherence to these guidelines. Despite this variation, research has consistently reported age- and sex-specific differences in FMD, where FMD is lower in males compared to females, and declines with age, even in healthy individuals. Reproductive hormones may explain sex-specific differences in FMD, with animal work suggesting that oestrogen improves the relationship between shear stress and vasodilation. Furthermore, exercise/heating protocols have demonstrated the potency of shear stress as a stimulus for improved FMD. The pattern of shear stress is also relevant, given that antegrade and retrograde shear stress impose opposite effects on FMD. However, these studies have manipulated mean shear stress, whilst it could be argued that fluctuations in blood flow and shear stress are more applicable to daily life. Therefore, the aims of this thesis were to: (i) update the expert-consensus guidelines for FMD, (ii) estimate age- and sex-specific reference values for brachial artery FMD in healthy individuals and explore the relation with CVD risk factors, (iii) examine age- and sex differences in the relation between FMD and its eliciting shear stress stimulus, and (iv) assess the acute effect of fluctuations in shear rate on FMD.

Chapter 3 aimed to update the expert-consensus methodological guidelines for the performance and analysis of FMD. Importantly, standardised performance of the FMD technique facilitates better between-study comparability, therefore reducing variability. This effort also importantly contributes to the construction of reference values (*Chapter 4*). Brachial artery FMD data (acquired according to protocol guidelines) and participant characteristics/medical history from 5,362 individuals (4-84yrs; 2,076 females) were pooled into a single database. Healthy individuals (n=1,403 [582 females]) were used to generate age-/sex-specific percentile curves from fractional polynomial regression. Subsequently, individuals with CVD risk factors, but without overt disease were included (un-medicated n=3,167 [1,247 females], and medicated n=792 [247 females]), and multiple linear regression tested the relation of CVD risk factors with FMD. Healthy males showed a negative, curvilinear relation between FMD and age, whilst females revealed a negative linear relation that started higher, but declined at a faster rate than males. Age- and sex-specific differences in FMD relate, at least partly, to baseline artery diameter. FMD was related to CVD risk factors in un-medicated (e.g. systolic-/diastolic blood pressure) and medicated individuals (e.g. diabetes/dyslipidaemia). Sex mediated

some of these effects (*P*<0.05), with normalisation of FMD in medicated men, but not women with dyslipidaemia.

To better understand the impact of sex and age on the relation between shear stress and FMD, healthy adults (n=932 [283 women]) were stratified into young adults (18-40 years, 389 men, 144 women) and older adults (>40 years, 260 men, 139 women). Secondly, women were grouped based on hormonal status (pre- [n=173] and post-menopausal [n=110]). There was evidence of a weak correlation between SRAUC and FMD in all groups but older men, although there was variation in strength of outcomes. Further exploration using interaction terms (age-sex*SRAUC) in linear regression revealed differential relationships with FMD (young women versus young men (β=-5.8-4, *P*=0.017) and older women (β=-5.9-4, *P*=0.049)). The correlation between SRAUC and FMD in pre-menopausal women (r^2=0.097) was not statistically different to post-menopausal women (r^2=0.025; Fisher: *P*=0.30). Subgroup analysis using stringent inclusion criteria for health markers (n=505) confirmed a stronger FMD-SRAUC correlation in young women compared to young men and older women.

Chapter 6 aimed to examine the impact of fluctuations in shear, whilst maintaining mean shear levels around baseline. Fifteen healthy males (27.3±5.0 years) underwent bilateral brachial artery FMD assessment before and after unilateral exposure to 30 minutes of intermittent negative pressure (10seconds -40mmHg, 7seconds 0mmHg) to induce fluctuation in shear rate, whilst the contra-lateral arm was exposed to a resting period. Negative pressure significantly increased shear rate, followed by a decrease in shear rate upon pressure release (both *P*<0.001). Importantly, across the 30-minute intervention, mean shear rate was not different compared to baseline (*P*=0.458). A linear mixed model revealed a significant effect of time for FMD (*P*=0.029), with exploratory post-hoc analysis showing an increase in the intervention arm (∆FMD +2.0%, *P*=0.008), but not in the contra-lateral control arm (∆FMD +0.5%, *P*=0.664). However, there was no effect for arm (*P*=0.619) or interaction effect (*P*=0.096).

Taken together, the work contained in this thesis successfully updated the FMD protocol guidelines and, for the first time, estimated reference values for brachial artery FMD. Firstly, the present findings highlight an important role for sex in determining vascular function. Specifically, sex altered the age-related decline in FMD and relation with some CVD risk factors and medication (*Chapter 4*), in addition to the relation between FMD and SRAUC (*Chapter 5*), suggesting improved sensitivity of the endothelium to shear stress in women. Secondly, novel data presented in *Chapter 6* suggest that fluctuations in shear pattern, even in the absence of altered mean shear, represents a stimulus to acute change in FMD in healthy individuals.

Declaration

I declare that the work contained within this thesis is entirely my own

Submitted manuscripts directly based on the work contained within this thesis

Holder, S.M., Bruno, R.M., Shkredova, D.A., Dawson, E.A., Jones, H., Hopkins, N.D., Hopman, M.T.E., Bailey, T.G., Coombes, J.S., Askew, C.D., Naylor, L., Maiorana, A., Ghiadoni, L., Thompson, A., Green, D.J., & Thijssen, D.H.J, on behalf of the TIFN International Working Group on Flow Mediated Dilation. (under review). *Age- and sex-specific reference intervals for brachial artery flow-mediated dilation in healthy individuals and the relation with cardiovascular disease risk factors.*

Holder, S.M., Dawson, E.A., Brislane, Á., Hisdal, J., Green, D.J., & Thijssen, D.H.J. (2019). *Fluctuation in shear rate, with unaltered mean shear rate, improves brachial artery flow-mediated dilation in healthy, young men.* Journal of Applied Physiology, 126(6), 1687-1693. doi: 10.1152/japplphysiol.00009.2019.

Thijssen, D.H.J., Bruno, R.M., Van Mil, A., **Holder, S.M.**, Faita, F., Greyling, A., Zock, P., Taddei, S., Deanfield, J.E., Luscher, T., Green, D.J., & Ghiadoni, L. (2019). *Expert consensus and evidence-based guidelines for the assessment of flow-mediated dilation (FMD) in humans.* European Heart Journal, 40(30), 2534-2547. doi: 10.1093/eurheartj/ehz350.

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Carter, S.E., Draijer, R., **Holder, S.M.**, Brown, L., Thijssen D.H.J., & Hopkins, N.D. (2019). *Effect of different walking break strategies on femoral artery endothelial function.* Physiological Reports, 7 (16), e14190. doi: 10.14814/phy2.14190.

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Holder, S.M., Dawson, E.A., Thompson, A., Green, D.J. & Thijssen. D.H.J. *Age and sex differences in vascular function: the role of oestrogen.* Pushing Boundaries: Women in Sport and Exercise, London, June 2019.

Holder, S.M., Dawson, E.A., Thompson, A., Green, D.J. & Thijssen, D.H.J. *Age, sex and vascular function: what's normal?* Liverpool John Moores University Institute for Health research café, May 2019.

Holder, S.M., Brislane, Á., Dawson, E.A., Green, D.J., & Thijssen, D.H.J. *The relation between shear stress and vascular function: the impact of sex.* Liverpool John Moores University Faculty of Science Postgraduate Research Day, June 2018.

Holder, S.M., Low, D.A., Graves, L.E.F., Carter, S.E., Brislane, Á., & Hopkins, N.D. *The effect of sedentary behaviour on cardiovascular biomarkers in active, healthy adults.* European College of Sport Science, Essen, Germany, July 2017.

Poster communications

Holder, S.M., Dawson, E.A., Brislane, Á., Hisdal, J., Green, D.J., & Thijssen, D.H.J. *An acute bout of intermittent negative pressure improves endothelial function in young, healthy males.* Northern Vascular Biology Forum, Bradford, December 2018.

Holder, S.M., Dawson, E.A., Green, D.J., & Thijssen, D.H.J. *Flow-mediated dilation: what's normal?* Liverpool John Moores University Faculty of Science Postgraduate Research Day, June 2017.

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Chapter 1

General Introduction

1.1 Background

Cardiovascular disease (CVD) remains the world's leading cause of morbidity and mortality (World Health Organisation, 2017). Atherosclerosis underlies the development of most forms of CVD and the vascular endothelium, the inner monolayer of cells of the arterial wall, plays a key role. A prime function of the endothelium is to regulate vascular tone via the synthesis and release of vasoactive substances (Cahill & Redmond, 2016). A major stimulus for this release of paracrine hormones is the frictional force of flowing blood, or shear stress, along the endothelium. One vasoactive substance released in response to augmented antegrade shear stress (forward; away from the heart) is nitric oxide (NO) (Palmer et al., 1988), which diffuses into vascular smooth muscle cells to cause relaxation and facilitate vasodilation. Accordingly, the vasodilator response is a reflection of an intact, functional endothelium (Ross et al., 1984). Previous studies have reported that endothelial dysfunction contributes to the development and progression of atherosclerosis (Charakida et al., 2010).

Several techniques have been developed over the past ~25 years to assess endothelial function. One frequently used non-invasive method relates to the flow-mediated dilation (FMD) approach. Briefly, this technique involves the use of high-resolution ultrasonography to measure conduit artery (most commonly the brachial artery) diameter in response to a marked elevation in blood flow and consequent shear stress following a period of distal limb ischaemia. FMD is reported as the relative change (presented as a percentage) in artery diameter from baseline. Previous research has provided evidence that the FMD response is endothelium-dependent (Dawson et al., 2010a) and largely mediated by NO (Green et al., 2014) in humans. In healthy individuals, one may expect marked vasodilation and the conceptual idea is that a low or absent dilation of the artery during the FMD test reflects endothelial dysfunction. Moreover, such an impaired dilator response, even in asymptomatic individuals, is related to CVD risk (Ras et al., 2013; Xu

et al., 2014; Inaba et al., 2010; Matsuzawa et al., 2015). Despite its valid conceptual basis, various factors influence the variability of FMD (De Roos et al., 2003; Donald et al., 2008), including lifestyle factors, hormonal changes, test protocol methodology and analysis method (Green et al., 2011; Thijssen et al., 2011a). Many of these factors are currently controlled by adopting expert-consensus guidelines (Thijssen et al., 2011a). The importance of this is supported by the fact that adherence to these guidelines is associated with a significantly lower variability of the FMD (Greyling et al., 2016a).

Since the introduction of the FMD technique in 1992, ample evidence has demonstrated the presence of age- and sex-specific differences in FMD in healthy individuals (Celermajer et al., 1994b; Taddei et al., 1996; Taddei et al., 1995; Adams et al., 1996; Yao et al., 2014; Hopkins et al., 2015; Gerhard et al., 1996; Juonala et al., 2008). Despite the general consensus in the literature regarding age- and sex-specific differences in FMD, methodological variations are present between studies which makes direct comparison difficult. Accordingly, ***aim 1*** of this thesis was to update the expert-consensus guidelines for the performance of the FMD. Secondly, a key limitation preventing the widespread use and interpretation of the FMD relates to the absence of reference values. Therefore, ***aim 2*** was to construct age- and sex-specific reference values for brachial artery FMD in healthy individuals and explore the relation with CVD risk factors.

Although the terminology of the FMD test implies that vasodilation is flow-mediated, experimental research has determined that the vasodilator response during reactive hyperaemia is mediated by the total post-deflation shear stress stimulus (Pyke & Tschakovsky, 2007). This is calculated as the area under the shear rate curve (SRAUC), from cuff release to peak diameter. However, SRAUC poorly correlates with the magnitude of FMD, and the strength of the relationship is age-dependent (Thijssen et al.,

2009a). Furthermore, previous work in animals suggests a positive effect of the sex hormone oestrogen in relation to the sensitivity to a given shear stress stimulus (Huang et al., 1998). This effect may explain the larger FMD observed in females compared to males, and also the larger FMD in pre-menopausal women compared to post-menopause. Nonetheless, this hypothesis has not been examined in humans. Therefore, ***aim 3*** of this thesis was to explore the relationship between FMD and SRAUC across the lifespan in healthy men and women, and also compare pre- *versus* post-menopausal women.

Shear stress plays a pivotal role in the pathophysiology of atherosclerosis, where areas of the vascular tree exposed to unidirectional shear stress are protected from atherosclerotic lesions (Cecchi et al., 2011). Due to the direct interaction with the endothelium, increasing the antegrade (towards the periphery) component of shear improves endothelial function (Tinken et al., 2009), whilst increased exposure to retrograde shear (backward; towards the heart) leads to a decrease in endothelial function (Schreuder et al., 2015; Thijssen et al., 2009c). In contrast to experimental conditions where prolonged exposure to unidirectional antegrade or retrograde shear is possible, daily living seems more related to fluctuations in antegrade and retrograde shear stress in response to variations in blood pressure and bodily movement completing everyday tasks. Although little previous work has focused on fluctuations in shear (opposed to prolonged unidirectional shear), some studies have demonstrated positive clinical effects of fluctuations in shear stress, such as improved wound healing in patients with lower limb ischaemia (Sundby et al., 2016a; Sundby et al., 2018b; Sundby et al., 2018a). These positive outcomes suggest that fluctuations in shear stress impact vascular health. However, these studies did not control for overall differences in antegrade or retrograde shear. Therefore, it is currently unknown whether fluctuations in shear stress *per se* influence FMD. Therefore, ***aim 4*** of this thesis was to assess the

acute effect of fluctuations in shear stress levels, with preservations on mean shear stress, on brachial artery FMD in humans *in vivo*.

1.2 Aims

The specific aims of this thesis were to:

1. Update the expert-consensus guidelines for the performance of the FMD (*Chapter 3*).
2. A) Construct age- and sex-specific reference values for brachial artery FMD in healthy individuals obtained with strict adherence to contemporary expert-consensus guidelines (*Chapter 4*).

   B) Examine the impact of cardiovascular risk factors on age- and sex-specific reference values for brachial artery FMD (*Chapter 4*).
3. Explore the impact of sex and female hormonal status on the relationship between FMD and SRAUC across the lifespan in healthy individuals (*Chapter 5*).
4. Assess the acute effect of fluctuations in shear stress, with preserved mean level of shear, on brachial artery FMD in healthy young men (*Chapter 6*).

Chapter 2

Literature Review: Age, Sex and Other Factors Influencing FMD

2.1 Cardiovascular Disease

Recent statistics show that 31% of global deaths were attributable to CVD (World Health Organisation, 2017), emphasising the importance of prevention, early detection and subsequent treatment of CVD. The underlying process of CVD is atherosclerosis, an inflammatory process affecting the arteries, whereby artery wall thickening occurs due to the build-up of substances such as cholesterol with vascular smooth muscle cells to ultimately lead to the formation of a plaque (Cahill & Redmond, 2016). If undetected/untreated, this process leads to partial blockage or sudden rupture of a plaque, which is clinically referred to as a myocardial infarction or stroke. Importantly, an impairment of the functionality of the vascular endothelium contributes to the development and progression of atherosclerosis (Juonala et al., 2004; Charakida et al., 2010; Chiu et al., 2009). Endothelial dysfunction has been identified as an early biomarker of CVD (Deanfield et al., 2007; Takase et al., 1998) and this can be detected at a very early stage of the atherosclerosis timeline, well before thickening of the arterial wall or early formation of atherosclerotic plaques (Figure 2.1).

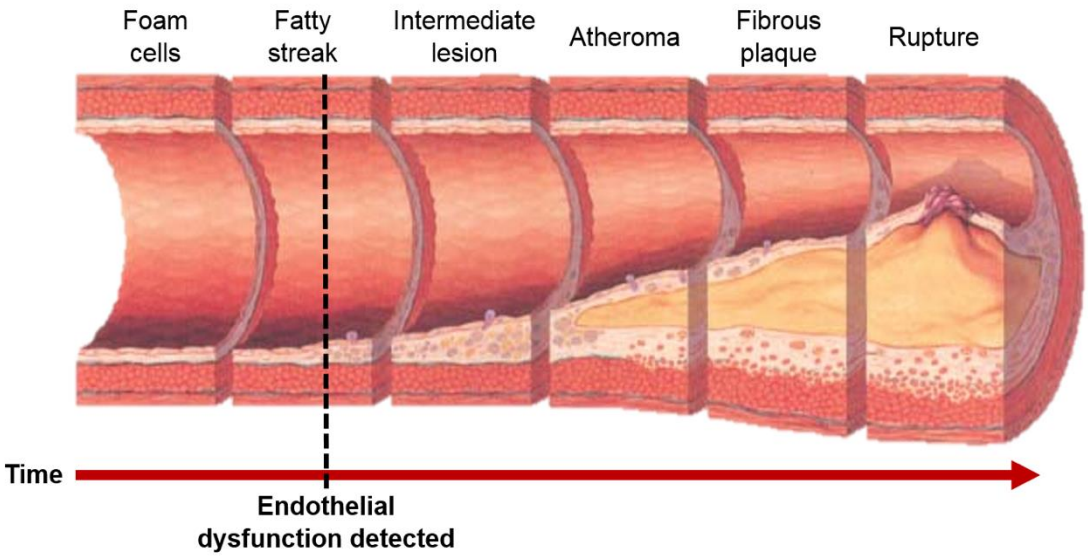

Figure 2.1: Timeline of the progression of atherosclerosis, adapted from Pepine (1998).

2.1.1 The Vascular Endothelium

The vascular endothelium is the inner layer of the vessel wall, lining the whole circulatory system, and its placement between the blood and artery wall facilitates interaction via cellular signalling. The endothelium is responsive to chemical (e.g., hormonal) and hemodynamic stimuli (mechanical forces related to flowing blood) and its function includes regulating platelet function, vascular smooth muscle cell growth and vascular tone (Cahill & Redmond, 2016). The most important hemodynamic stimulus for dilation and adaptation is shear stress (frictional force of flowing blood against the endothelium). Increased shear stress activates mechanosensors located within the endothelial cells (Labrador et al., 2003), which in turn initiates a cascade of signalling pathways (Chien, 2007). This results in the synthesis and release of the vasodilators (e.g. NO, endothelium-derived relaxing factor (EDRF) and prostaglandins) from the endothelium (Bloodsworth et al., 2000). Vasodilation occurs to accommodate for the increase in blood flow, and a marked dilator response is reflects a healthy endothelium (Ross et al., 1984).

2.1.2 Hemodynamic Laws of Blood Flow

Blood flow is influenced by multiple physiological factors, including blood pressure, vessel radius and length, and blood viscosity. The relationship between these variables were examined by Jean Poiseuille by passing water through glass tubes (of varying diameters and lengths), with different pressures applied to one end (Poiseuille & Herschel, 1940; Pfitzner, 1976). As a result of this work, he developed an equation, now known as Poiseuille's Law (Pfitzner, 1976):

$$V = \frac{\pi \mathrm{p} \mathrm{r}^4}{8 \eta \mathrm{l}}$$

where V = flow rate; p = pressure gradient; r = radius; η = viscosity; l = length.

The most important factor determining blood flow relates to pressure. In physics, fluid flows through a tube from an area of high- to low pressure, with the difference in pressure known as the pressure gradient. In the body, pressure originates from contraction of the heart as the driving force of blood around the circulatory system. Additionally, the pressure gradient is multiplied by the radius^4, highlighting the importance of artery diameter as a determinant of blood flow. Another mediator of blood flow is viscosity (i.e. thickness of the blood). Importantly, viscosity is affected by age, sex, menstrual cycle phase and some CVD risk factors (Tremblay, 2019), and is a factor in the calculation of shear stress. However, despite the association with variables mentioned above, viscosity is largely ignored and assumed to be constant in FMD research (Parkhurst et al., 2012).

2.1.3 Nitric Oxide

A Nobel prize-winning discovery by Furchgott & Zawadzki (1980) (more by serendipity than design) demonstrated *in vitro* that acetylcholine administration failed to cause relaxation in blood vessels with the endothelial cells rubbed off, whilst vasodilation was observed when the endothelium was intact. This vasodilation response was mediated by the release of vasoactive substances, namely EDRF and NO. This landmark study initiated the recognition of the pivotal role of the endothelium in the regulation of vascular tone and vasomotor function, but also the importance of NO in vascular health. NO is released basally (Vallance et al., 1989) and is synthesised by the enzyme eNOS from the amino acid L-arginine (Palmer et al., 1988). NO possesses a myriad of anti-atherogenic properties to maintain vessel homeostasis, including inhibition of cell growth and proliferation, leukocyte and platelet adhesion, regulation of vascular tone and antithrombotic properties (Bloodsworth et al., 2000). Traditional CVD risk factors such as hypertension, smoking and obesity are associated with attenuated NO levels (Yetik-Anacak & Catravas, 2006), whilst physical activity or exercise is believed to increase NO bioavailability (Schuler et al., 2013). These established relationships emphasise the

importance of the NO pathway for the endothelium and for cardiovascular health and disease.

2.1.4 Endothelial Function

Endothelial function refers to the sensitivity and ability of the endothelium to regulate vascular tone in response to various stimuli and is essential for vascular health (Green et al., 2004). Endothelial dysfunction is associated with greater CVD risk (Deanfield et al., 2007), even in asymptomatic individuals (Ras et al., 2013; Xu et al., 2014; Inaba et al., 2010; Matsuzawa et al., 2015). Based on the detrimental role of endothelial dysfunction in the atherosclerotic process, studies have attempted to develop and validate non-invasive methods and biomarkers to assess endothelial function in humans *in vivo*. Celermajer et al. (1992) were the first to develop and utilise the non-invasive technique now known as the FMD test. This measurement simply refers to the assessment of a conduit artery dilator response to an increase in blood flow which is endothelium-dependent (Dawson et al., 2010a) and partly mediated by NO (Green et al., 2014). FMD is most commonly assessed in the brachial artery due to its feasibility and relatively easy access compared to other conduit vessels (e.g. radial or femoral artery), with studies emphasising the independent predictive value of this test for future CVD risk (Matsuzawa et al., 2015). Therefore, the focus of this thesis will be on FMD performed in the brachial artery.

2.2 Flow-Mediated Dilation

2.2.1 Prognostic Value

The measurement of endothelial function using FMD has become a popular research tool, likely due to its non-invasive nature, ability to predict cardiovascular events (Inaba

et al., 2010; Ras et al., 2013; Vita & Keaney, 2002; Thijssen et al., 2011a) and correlation to coronary artery endothelial function (Anderson et al., 1995; Takase et al., 1998; Broxterman et al., 2019). Several meta-analyses have explored the prognostic value of FMD, and revealed that a 1% increase in brachial artery FMD is associated with an 8-13% reduction in CVD risk in heterogeneous populations (Ras et al., 2013; Inaba et al., 2010; Matsuzawa et al., 2015). Despite the association between FMD and CVD risk, FMD has failed to add to the value of traditional risk factors for net reclassification index of risk (Peters et al., 2012) compared to the Framingham Risk Score (Yeboah et al., 2012).

The FMD technique is widely used in clinical studies, given that endothelial function rapidly responds to interventions such as pharmacological substances (Luscher et al., 2012) and exercise training (Green & Smith, 2017). The relevance of this is that individuals can be identified as "non-responders", or pharmacological agents deemed ineffective, and therefore interventions can be altered as required. For example, FMD was the primary outcome in the dal-VESSEL clinical trial (Luscher et al., 2012), examining the vascular effects of a cholesterol-lowering drug. The authors found that whilst the drug was successful in reducing low-density lipoprotein (LDL) and increasing high-density lipoprotein (HDL) cholesterol, FMD was not affected. This evidence demonstrated no negative vascular effects of the drug, therefore highlighting its safety for future research. Despite this potency, the FMD lacks clinical applicability as normal and reference values have yet to be established.

2.2.2 Endothelium-Dependent Dilation

FMD represents the relative change in conduit artery diameter in response to distal limb ischaemia. The current FMD test protocol consists of measurement of one minute of baseline artery diameter and blood flow recording using high-resolution ultrasound, followed by a 5-minute period of distal limb ischaemia using a pneumatic cuff (inflated to a suprasystolic pressure) around the forearm. Following cuff release, artery diameter and blood flow are recorded for a further 3 minutes (Thijssen et al., 2011a). Subsequently, FMD is calculated as the relative (percentage) change in diameter from baseline to peak following cuff release. The underpinning physiology of the FMD test is depicted in Figure 2.2.

The endothelium-dependency of the vasodilation response to an increase in shear stress has been reviewed elsewhere (Green et al., 2017). In brief, studies in both animals (Berdeaux et al., 1994; Pohl et al., 1986) and humans (Dawson et al., 2010b) have shown an attenuated or even abolished dilation response to increased shear stress with endothelial denudation (i.e. removal of the endothelium). Furthermore, given the role NO (produced in the endothelial cells) in the FMD (Green et al., 2014), an intact endothelium is essential for vascular function and health.

2.2.3 Endothelium-Independent Dilation

Independent of the functionality of the endothelium and the magnitude of the eliciting shear stress stimulus, the ultimate vasodilation response is reliant upon functional vascular smooth muscle cells (Komai et al., 2008). For this reason, brachial artery diameter is continuously assessed at baseline and in response to an exogenous NO donor, glyceryl trinitrate (GTN), administered via sublingual spray or tablet. GTN directly relaxes vascular smooth muscle cells and, therefore, can be considered as an

endothelium-independent vasodilation. The original FMD paper by Celermajer et al. (1992) showed an impaired GTN response in smokers and coronary artery disease patients compared to healthy adults, observations which have been supported by others (Zhang et al., 2000; Raitakari et al., 2001). This suggests that the impaired FMD in smokers and subjects with coronary artery disease could, at least partly, be explained through an impaired ability of vascular smooth muscle cells to mediate vasodilation. Furthermore, impaired GTN responses are associated with traditional modifiable CVD risk factors (Celermajer et al., 1993; Adams et al., 1998; Gokce et al., 2001) as well as age (Montero et al., 2015) and sex (Black et al., 2009). Evidence suggests that endothelial- and vascular smooth muscle dysfunction occur at different time points along the atherosclerosis timeline (Adams et al., 1998; Kawano et al., 2012), whereby endothelial dysfunction precedes vascular smooth muscle dysfunction, with the latter being prevalent with increasing severity of the disease/risk factor. Given the relationship between GTN response, FMD and traditional (modifiable and non-modifiable) CVD risk factors, vascular smooth muscle cell function remains an important component contributing to conduit artery vasodilator capacity.

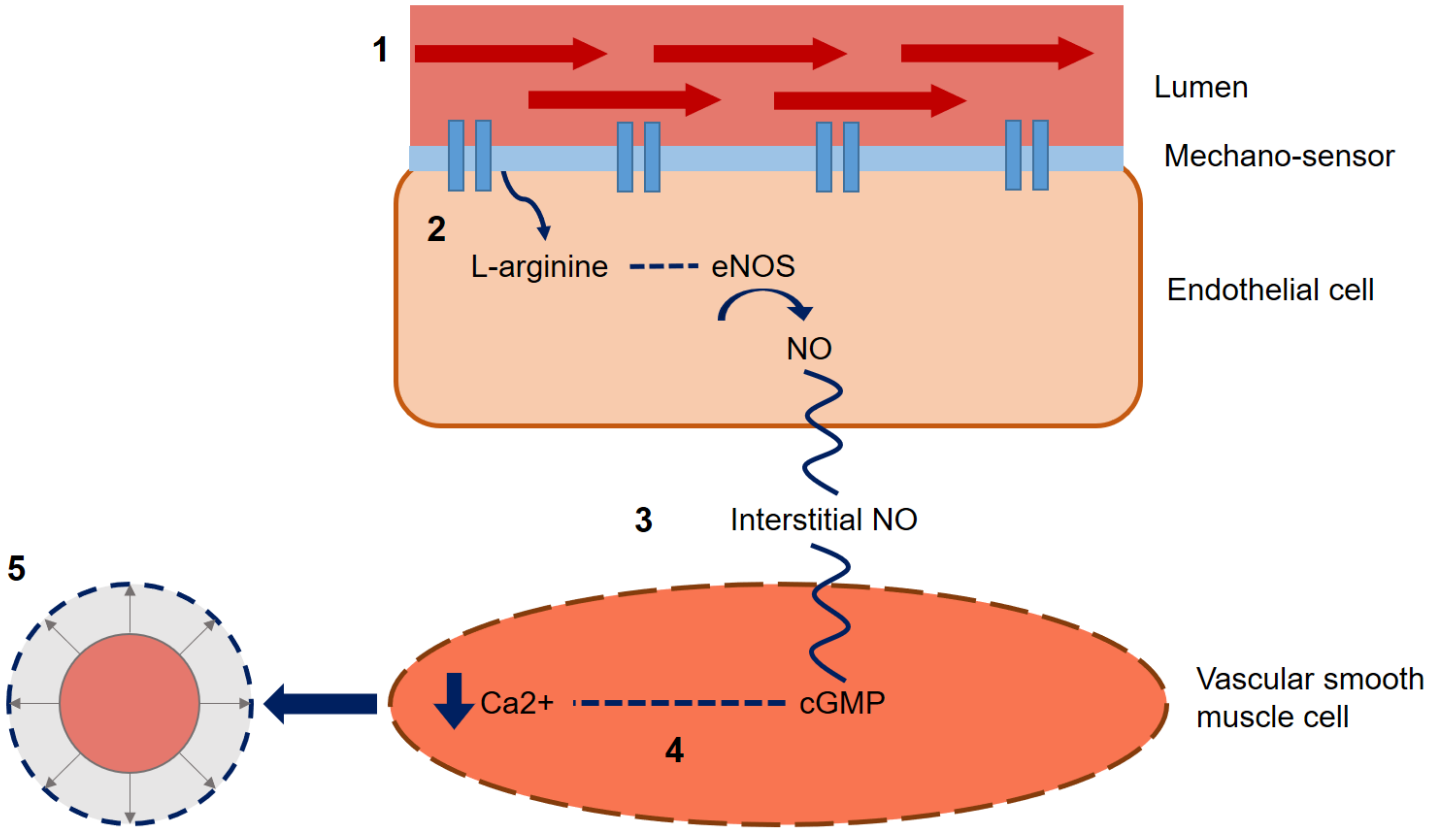

Figure 2.2: Schematic representation of the physiology behind the FMD. Step 1: Shear stress stimulus following cuff release is sensed by mechano-sensors. Step 2: Shear stress mechanotransduction initiates signalling cascade and subsequent vasodilator production. Step 3: Vasodilator diffusion from endothelium into vascular smooth muscle cell. Step 4: Signalling transduction cascade results in reduced calcium concentration and smooth muscle relaxation. Step 5: Vasodilation.

2.3 Ageing and FMD

Advancing age is an established risk factor for CVD. Ample experimental evidence shows an age-related decline in brachial artery endothelial function across the lifespan (Brandes et al., 2005; Seals et al., 2011), even without the presence of CVD risk factors (Gerhard et al., 1996; Celermajer et al., 1994b; Taddei et al., 1996; Taddei et al., 1995; Tschudi et al., 1996). The first research group to quantify brachial artery FMD changes with age were Celermajer et al. (1994b) in over 200 healthy participants. Interestingly, the authors observed that FMD was maintained in females until around 50 years, coinciding with the onset of menopause, whilst men showed a lower FMD, and an earlier decline around 40 years (Figure 2.3).

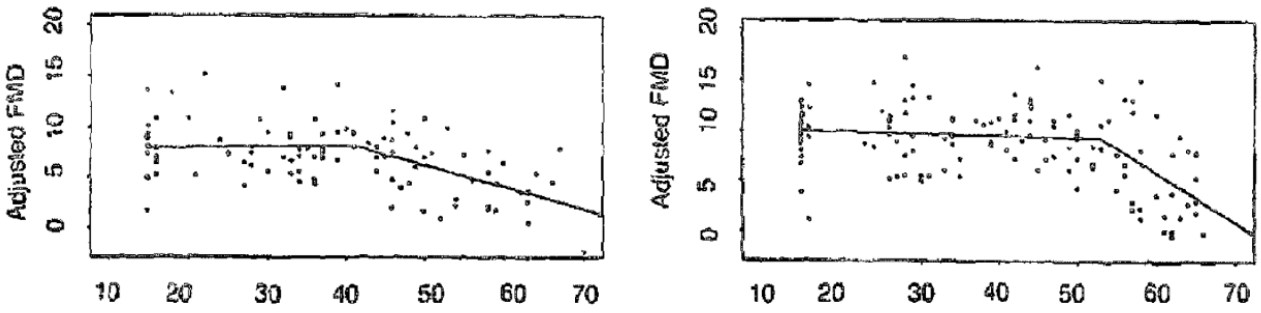

Figure 2.3: FMD changes with age in men (left) and women (right), from Celermajer et al. (1994b).

On a larger scale, a population-based study by Skaug et al. (2013) assessed the age-related differences in brachial artery FMD in 4739 adults and observed a progressive decline in FMD until age 70 and 80 years in men and women, respectively. However, the authors measured the peak FMD diameter 60-seconds post cuff deflation, a technical error with potentially significant implications for the validity of the conclusion (which is further discussed in *Chapter 3*). Furthermore, whilst participants in this cohort were defined as healthy, smokers were included who are established to have impaired FMD compared to healthy controls (Celermajer et al., 1992). Despite the methodological

issues, a trend for decline in FMD with advancing age exists. Various age-specific physiological alterations may underlie this relationship.

2.3.1 Mechanisms of Impaired FMD with Ageing

2.3.1.1 Changes in Artery Structure

The key structural variations associated with age are related to artery wall thickening (i.e. increasing intima-media thickness; IMT) and increased arterial stiffness. Carotid artery IMT is considered a surrogate marker of atherosclerosis and predictor of cardiovascular events (O'Leary et al., 1999). Importantly, age- and sex-specific reference intervals have been established for carotid artery IMT (Engelen et al., 2013), whereby IMT increases with healthy ageing. Furthermore, IMT was inversely associated with FMD (Juonala et al., 2004; Yao et al., 2014), even in an asymptomatic population (Halcox et al., 2009). As described earlier, endothelial dysfunction is detected at a very early stage of the atherosclerosis timeline, before thickening of the artery wall becomes evident (Figure 2.1). This highlights the importance of assessing endothelial function for early detection of CVD, prior to observable structural changes.

Arterial stiffness, which can be measured using pulse wave velocity, is a recognised risk factor and predictor for CVD morbidity and mortality (Yambe et al., 2004; Vlachopoulos et al., 2010). Stiffening of the artery can be a result of changes in the structural and cellular properties of the artery wall (Zieman et al., 2005), and is commonly associated with ageing (Lakatta & Levy, 2003; Bossuyt et al., 2015; Engelen et al., 2015). Related to endothelial function, a stiffer artery (via reduced elasticity) limits the dilatory capacity and therefore FMD. Limited evidence is available regarding the inter-relationships between pulse wave velocity, FMD and age. However, increased stiffness of conduit

arteries with older age could mediate the inverse association observed between pulse wave velocity and FMD (Heldens et al., 2013).

2.3.1.2 Nitric Oxide Bioavailability

Given the importance of the NO-pathway in FMD (Green et al., 2011), a reduction in NO is considered one of the largest contributors to the age-related impairment of FMD. This is partially due to the attenuated ability of endothelial cells to generate NO with age (Al-Shaer et al., 2006), through a natural age-related decline in eNOS expression and activity (Tschudi et al., 1996; Tanabe et al., 2003), even in asymptomatic individuals (Toda, 2012). An elegant study by Taddei et al. (2001) demonstrated a progressive decline in (acetylcholine-induced) endothelium-dependent vasodilation with age. Interestingly, there appeared to be no association between age and (sodium nitroprusside-induced) endothelium-independent vasodilation, which implies that the decline in the endothelial function is, at least partly, mediated by the progressive impairment of the L-arginine-NO pathway.

2.3.1.3 Oxidative Stress

Oxidative stress is determined by an imbalance in pro- and anti-oxidant enzymes in the blood (Lessiani et al., 2016), more specifically the excessive production of reactive oxygen species, with an insufficient anti-oxidant defence system (free radical scavenging) (Seals et al., 2014). Oxidative stress plays a key role in the atherosclerotic process (Witztum, 1994; Kattoor et al., 2017; Summerhill et al., 2018) and is considered to be a key determinant contributing to age-related endothelial dysfunction (Finkel & Holbrook, 2000; Harman, 2003; Donato et al., 2007; Taddei et al., 2001; Donato et al., 2018). With increasing age, oxidative stress is associated with pro-inflammatory markers including adhesion molecules and cytokines (El Assar et al., 2013). This is largely due to the

negative impact of oxidative stress on NO bioavailability (Ungvari et al., 2010), and is a consequence of the loss of protective properties of NO outlined in *Section 2.1.2*. Specifically, experimental work demonstrated that administration of antioxidant vitamin C improved endothelial function in healthy older individuals (over 60 years old), but was ineffective in adults under 60 years old (Taddei et al., 2001). Donato et al. (2007) investigated the mechanisms associated with oxidative stress, endothelial function and ageing. The authors found increased expression of oxidative stress markers in the endothelial cells of healthy old men, compared to young men, with no age-related differences in anti-oxidant enzyme expression, demonstrating an imbalance between pro- *versus* anti-oxidant enzymes in older individuals. Additionally, these markers were inversely related to brachial artery FMD, implying an important role of oxidative stress in the age-related decline in endothelial function (Donato et al., 2007).

Further work has demonstrated that the association between oxidative stress and endothelial dysfunction is enhanced by the presence of CVD risk factors such as hypertension (Taddei et al., 1998; Harvey et al., 2015). Multiple clinical studies in hypertensive patients have shown a positive correlation between blood pressure and oxidative stress (Mihalj et al., 2016; Rodrigo et al., 2007; Lacy et al., 2000; Redon et al., 2003; Touyz, 2004). Others have also shown a simultaneous increase in inflammatory marker expression (Herrera et al., 2010; El Assar et al., 2013) in asymptomatic older individuals (Bottino et al., 2015), further suggesting a potential relationship between these processes. Based on the ability of free radicals to scavenge NO (Incalza et al., 2018), and the key role of the NO-pathway in mediating endothelium-dependent vasodilation, age-related increase in oxidative stress could contribute to blunted NO-mediated dilation and subsequent endothelial dysfunction with ageing.

2.3.1.4 Hemodynamic Changes: Forces

2.3.1.4.1 Parallel Force: Shear Stress

A typical shear pattern at rest is characterised by a large amount of antegrade shear during systole, followed by (in most cases) a brief period of retrograde shear during diastole (Figure 2.4). Disturbances in laminar blood flow and shear stress are associated with the atherosclerotic process, through the upregulation of pro-inflammatory genes and increased expression of factors including adhesion molecules and vasoconstrictors (Chiu et al., 2009). Older age is associated with elevated levels of retrograde and oscillatory shear stress in peripheral conduit arteries (Credeur et al., 2009; Young et al., 2010; Padilla et al., 2011; Casey et al., 2012), which is related to pro-atherogenic factors (Laughlin et al., 2008; Thijssen et al., 2009c; Newcomer et al., 2011; Schreuder et al., 2015), such as diminished NO bioavailability (Padilla et al., 2011). Proposed factors mediating an increase in retrograde and oscillatory shear stress include increased stiffness (Padilla et al., 2011), sympathetic nerve activity (Davy et al., 1998; Thijssen et al., 2014; Thijssen et al., 2006; Casey et al., 2012) and decreased vascular smooth muscle reactivity (Al-Shaer et al., 2006).

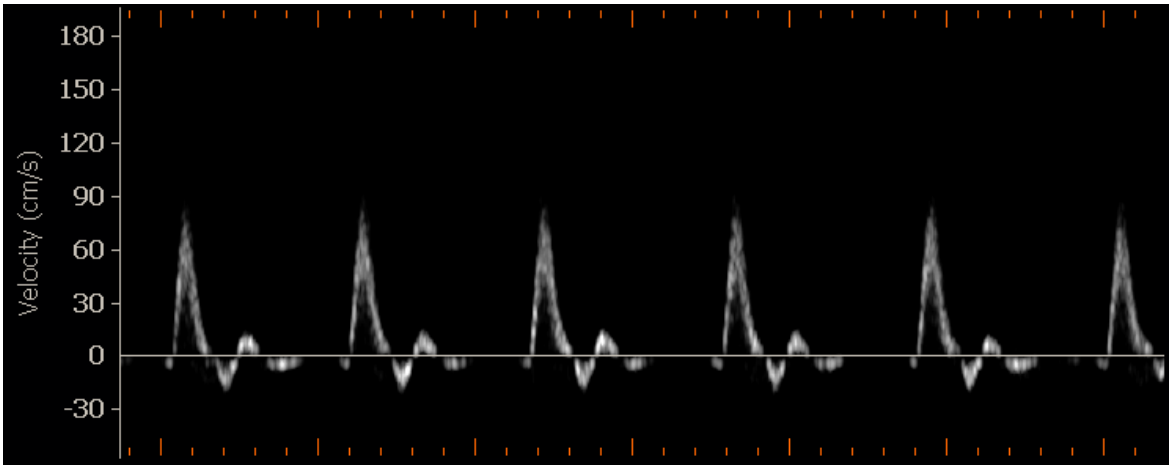

Figure 2.4: Blood flow velocity trace in the brachial artery of a healthy individual.

2.3.1.4.2 Perpendicular Force: Blood Pressure

Age-related increases in blood pressure are likely due to structural variances within the vasculature (Pinto, 2007), combined with inflammation and oxidative stress (Harvey et al., 2015) in the pathophysiology of hypertension. Whilst systolic blood pressure appears to be inversely related to brachial artery FMD (Benjamin et al., 2004), the implications of hypertension on FMD are discussed in *Section 2.5.3.2*. Research is limited on the effects of ageing on FMD within the normal blood pressure range, which therefore warrants further investigation.

2.3.1.5 Ageing and FMD: Summary

An age-related decline in FMD is considered “normal”, even in asymptomatic individuals. This may be mediated by a number of factors, contributing to structural and biochemical changes within the vascular wall. Hemodynamic changes, NO bioavailability, and subsequent vascular remodelling may be key mediators of endothelial dysfunction with age. However, a gap in our current knowledge remains regarding what is normal across the lifespan. Furthermore, there is a sex-specific difference in the change in FMD with ageing (Figure 2.3; Celermajer et al. (1994b)), and this will be discussed below.

2.4 Sex Differences in FMD

Sex-related differences in FMD have been reported since the early stages of FMD research (Adams et al., 1996; Celermajer et al., 1994b), demonstrating larger FMD in females compared to males. These observations have since been supported by multiple research groups (Juonala et al., 2008; Yao et al., 2014; Hopkins et al., 2015). The physiological differences between males and females are mostly determined by the difference in reproductive hormones, of which the influence will be discussed below.

2.4.1 Hormonal Fluctuations and Influences on FMD

2.4.1.1 Oestrogen and the Menstrual Cycle

Oestrogen is commonly believed to have cardio-protective effects against CVD, given the lower incidence of CVD in pre-menopausal women compared to age-matched men (Townsend et al., 2015a). The presence of oestrogen has also been shown to enhance endothelial function through the mediation of vasoactive substances, i.e., upregulation of vasodilators NO, prostacyclin and angiotensin 1-7, and attenuation of vasoconstrictor endothelin-1 (Novella et al., 2019). Underlying mechanisms of these effects include increased eNOS expression and activity (Hayashi et al., 1995; Sumi & Ignarro, 2003) and decreased oxidative stress (Wassmann et al., 2001; Dantas et al., 2002). Other positive vascular effects of oestrogen relate to regulation of inflammation (Bowling et al., 2014), circulating lipids (Muesing et al., 1996), and inhibition of endothelial cell apoptosis, vascular smooth muscle cell migration and proliferation (Weiner et al., 1994). Together, these effects contribute to the protection against the development of atherosclerosis and CVD (Mendelsohn, 2000).

The female sex hormones fluctuate throughout a normal menstrual cycle and oestrogen reaches its highest peak in the late follicular phase with a lower peak in the luteal phase (Figure 2.5). Previous studies have shown that FMD reflects fluctuating oestrogen levels during the menstrual cycle (Figure 2.6) (Williams et al., 2001; Brandão et al., 2014; Hashimoto et al., 1995; Adkisson et al., 2010; English et al., 1998; Kawano et al., 1996). More specifically, peaks in oestrogen levels matches with higher levels of FMD within individuals. Despite these observations, other groups have failed to show changes in FMD across the menstrual cycle (Shenouda et al., 2018; Saxena et al., 2012; Rakobowchuk et al., 2013; D'Urzo et al., 2018). This may relate to heterogeneity in methodology and therefore limit comparability between studies.

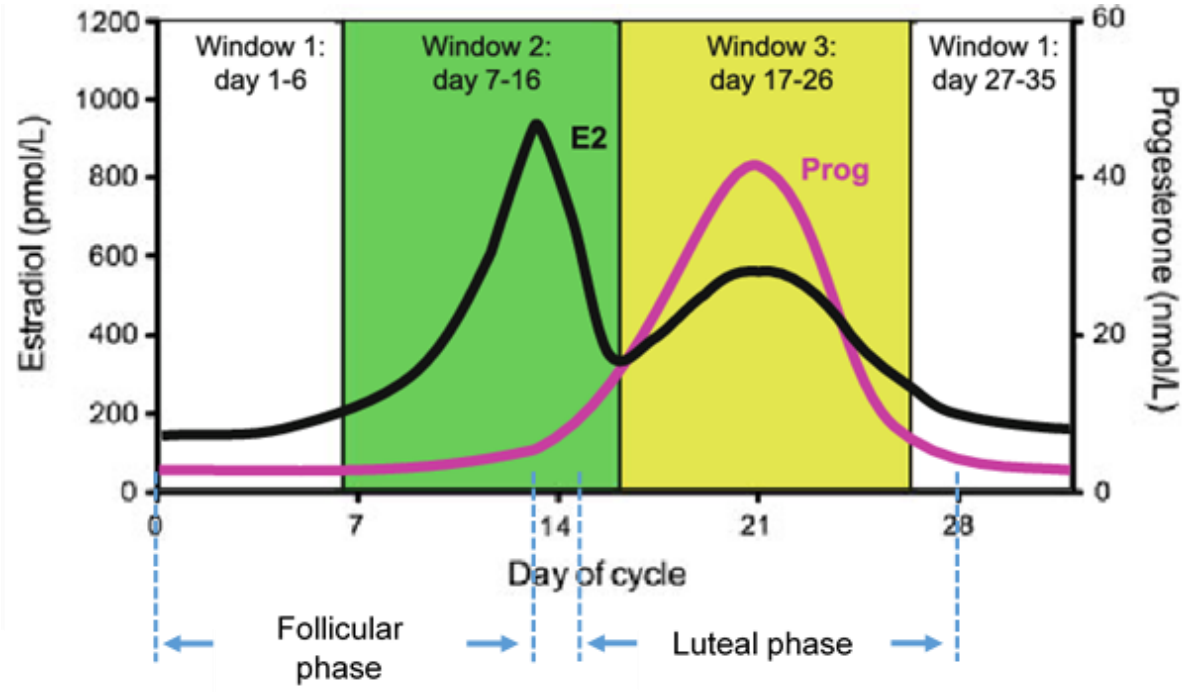

Figure 2.5: Fluctuating female sex hormone concentrations during the menstrual cycle adapted from Haynes et al. (2013). E2 – estradiol (oestrogen); Prog – progesterone.

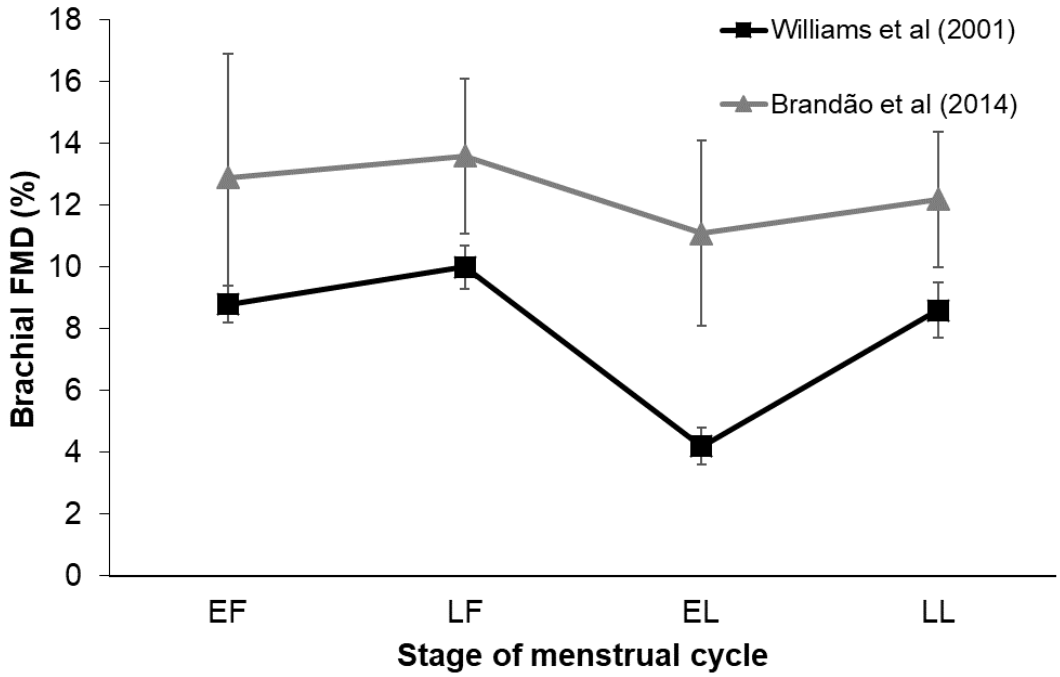

Figure 2.6: Change in brachial artery FMD throughout the stages of the menstrual cycle. EF – early follicular; LF – late follicular; EL – early luteal; LL – late luteal. Combined data from Williams et al. (2001) and Brandão et al. (2014).

Oestrogen receptors alpha and beta (ORα and ORβ, respectively) are expressed within the endothelial and vascular smooth muscle cells (Mendelsohn & Karas, 1999), and play an important role in mediating the vascular effects of oestrogen on the endothelium (Miller & Duckles, 2008). As such, it has been shown in cell culture, animal and human models that oestrogen mediates ORα expression (Ihionkhan et al., 2002; Pinna et al., 2008; Gavin et al., 2009; Sakaguchi et al., 2003), which in turn regulates eNOS activity (Hayashi et al., 1995; Chen et al., 1999). Gavin et al. (2009) measured brachial artery FMD, ORα expression, and eNOS expression and activation in healthy post-menopausal women, compared to pre-menopausal women during the early- and late follicular phase of the menstrual cycle, representing low and high levels of oestrogen, respectively (Figure 2.5). In line with previous work, the authors observed a lower ORα expression in the early follicular phase and post-menopause, consistent with lower circulating oestrogen, in addition to moderate positive correlations between brachial artery FMD with ORα expression, and ORα expression with eNOS. This is relevant since the action of an oestrogen molecule binding to a receptor activates eNOS, causing upregulated NO release (Russell et al., 2000). This action, combined with NO released in response to elevated shear stress (i.e. during the FMD test), and therefore greater NO abundance, may contribute to the larger FMD observed in pre-menopausal women (and during the late follicular phase) compared to post-menopausal women and age-matched men. Together these findings demonstrate the important physiological effects of oestrogen on vascular endothelial function, which may partially explain the cardio-protection of oestrogen in pre-menopausal women compared to men.

2.4.1.2 Testosterone

The role of testosterone on vascular function in men is poorly studied with conflicting results. Small studies in body builders (Ebenbichler et al., 2001; Sader et al., 2001) and hypogonadal men (Sader et al., 2003; Bernini et al., 2006) showed that testosterone

administration negatively affected FMD, suggesting an inhibitory effect of testosterone on endothelial function. Conversely, other studies have shown positive acute effects of testosterone on FMD (Ong et al., 2000) and coronary artery vasodilation (Webb et al., 1999) in coronary heart disease patients. Furthermore, population-based studies have found a positive relationship between testosterone and FMD (Empen et al., 2012; Akishita et al., 2007), whilst no association was observed for GTN response (Empen et al., 2012), suggesting an interaction between testosterone and the endothelium but not smooth muscle cell function. Interestingly, this relationship remained after statistically controlling for confounding variables including age, smoker status and other CVD risk factors, implying an independent relationship. Given the large number of factors influencing these previous studies, but also the conflicting results, further research is required to isolate the testosterone-FMD relationship.

2.4.2 Puberty

Puberty is characterised by the influx of sex hormones, representing a biological transition from childhood to adulthood (Day et al., 2015). This process generally occurs between age 8-13 years in girls, and 9-14 years in boys (Sorensen et al., 2012). Pubertal development is assessed clinically by Tanner stage on a scale from 1 (pre-pubertal) to 5 (post-pubertal) for boys and girls (Tanner & Whitehouse, 1976), including criteria for pubertal hair, menarche (onset of menstrual cycle) for girls, and deeper voice/facial hair for boys. Importantly, there is large between-individual variation in timing of the onset of puberty (Sorensen et al., 2012), with early age of menarche associated with greater CVD risk in females (Day et al., 2015). Physiologically, the surge in sex hormones has been found to be associated with changes in CVD risk factors, including body composition, systolic blood pressure and lipids (Siervogel et al., 2003; Moran et al., 2008; Marlatt et al., 2013). Interestingly, Moran et al. (2008) reported sex-specific differences in the rate of change of these factors during adolescence. More specifically, systolic blood pressure

and lean body mass increased with age at a greater rate in males compared to females. Additionally, body fat percentage and HDL cholesterol increased with age in females, but decreased in males. Research related to endothelial function during puberty is scant, with evidence of no difference in FMD between Tanner stages, despite differences in baseline artery diameter (Marlatt et al., 2013), implying no effect of pubertal maturation on endothelial function. In contrast to these findings, brachial artery FMD declined with age in male children and adolescents (age 6-18 years), whilst no pattern was evident in females (Hopkins et al., 2015). Nonetheless, the above studies are limited due to the lack of data related to hormone levels. Therefore, further research is warranted in children and adolescents to establish the true vascular effects of the puberty-related surge in sex hormones.

2.4.3 Menopause

Menopause is defined by the permanent cessation of menses (Bechlioulis et al., 2009), resulting in the loss of endogenous oestrogen. This generally occurs in women between the age of 45-55 years (Gold, 2011) and is associated with a decline in vascular endothelial function (Celermajer et al., 1994b; Taddei et al., 1996; Gavin et al., 2009; Moreau et al., 2012a) and accelerated CVD risk (Collins et al., 2007). Statistics from the British Heart Foundation show an exponential increase in deaths in women, coinciding with the onset of menopause (Townsend et al., 2015c). Celermajer et al. (1994b) observed a steep decline in womens' FMD response around menopausal age (Figure 2.3), whilst no changes in endothelium-independent dilation (GTN) were observed, suggesting that the decline in function was attributed to dysfunction of the endothelial cells. In addition, it has recently been reported that levels of retrograde and oscillatory shear stress increased across the menopause transition (Somani et al., 2019). These observations may be associated with downstream vasoconstriction and increased vascular resistance, induced by elevated muscle sympathetic nerve activity in post-

menopausal women (Hart et al., 2012). This proposed mechanism was supported by experimental work demonstrating increased retrograde shear rate with concomitant increases in muscle sympathetic nerve activity (Padilla et al., 2010). From the above evidence, oestrogen deficiency is a likely mediating mechanism behind the (post-menopausal) age-related decline in FMD in females.

Some post-menopausal women use hormone replacement therapy (HRT) to offset menopausal symptoms such as hot flushes. Given the cardio-protective role of oestrogen, one may expect HRT to influence endothelial function and subsequent CVD risk. Nonetheless, there is inconsistency within the current literature regarding the benefits and/or effectiveness of HRT on CVD risk (Bechlioulis et al., 2009). Whilst some research groups have demonstrated an improvement in FMD with oestrogen treatment (Moreau et al., 2012b; Moreau et al., 2013), various factors including the time between the onset of menopause and administration of hormone treatment (Vitale et al., 2008; Lobo, 2017), age (Sherwood et al., 2007), hormone (dosage and type) administered and FMD methodology likely mitigate the potential beneficial effects of HRT on vascular function. Overall, the effect of HRT on endothelial function and future CVD risk remains unclear as there appears to be considerable confounding factors, potentially influencing the effects of HRT in this population.

2.4.3 Sex Hormones: Summary

The protective role of oestrogen against the development of CVD appears to be modulated by the preservation of NO bioavailability via the L-arginine-NO pathway (Blumenthal et al., 2007). Whilst FMD may not be statistically different between males and females in the menstrual phase, female FMD was still greater (Hashimoto et al., 1995), which suggests that hormonal differences may not be solely responsible for the

sex-related difference in FMD. In general, females have a ~25% smaller artery diameter than males across all age ranges (Adams et al., 1996; Levenson et al., 2001; Hu et al., 2008; Juonala et al., 2008; Black et al., 2009). Since a smaller baseline diameter is associated with a larger FMD, some of the differences between sexes may be explained through the baseline artery diameter (Adams et al., 1996).

2.5 Other Influencing Factors on FMD

2.5.1 Baseline Diameter

For mathematical reasons, baseline artery diameter has a direct influence on FMD (i.e., FMD is calculated by dividing the absolute change in diameter by baseline diameter), and is therefore a strong predictor of the magnitude of FMD (Thijssen et al., 2009a). The statistical implications of this are discussed in *Chapter 3*. It is well documented that an inverse relationship exists between baseline artery diameter and FMD response in heterogeneous populations (Herrington et al., 2001; Thijssen et al., 2008b; Joannides et al., 2002; Thijssen et al., 2008a; Schroeder et al., 2000). A potential mediator of this relationship relates to the eliciting shear stress stimulus. Due to the inclusion of artery diameter within the calculation of shear rate, the total shear rate stimulus during reactive hyperaemia is greater in smaller arteries (Silber et al., 2001; Nishiyama et al., 2007; Parker et al., 2006b). Whilst differences between large and small vessels cannot be simply explained by shear alone (Thijssen et al., 2011b; Jazuli & Pyke, 2011), structural wall characteristics, hemodynamic forces and possible differences in hydrostatic pressures (e.g. upper *versus* lower limb arteries (Thijssen et al., 2011b)) may also influence the magnitude of the FMD response.

2.5.2 Eliciting Shear Stress Stimulus

Given the ability of shear stress to directly influence the endothelium, shear stress has become the focus of many research groups to understand its role in vascular (patho)physiology. The initial studies were performed in animals, which together demonstrated that elevation in shear stress leads to an endothelium-dependent dilation (Pohl et al., 1986; Berdeaux et al., 1994) and release of EDRF (Rubanyi et al., 1986). Many experimental studies have demonstrated a dose-dependent relationship between shear stress and vasodilation (Carter et al., 2013; Pyke & Tschakovsky, 2007; Pyke & Tschakovsky, 2005; Pyke et al., 2004; Atkinson et al., 2015b; Leeson et al., 1997). Specifically related to the FMD, Pyke & Tschakovsky (2007) explored the contribution of the post cuff deflation shear stress stimulus to the magnitude of vasodilation. Using cuff occlusion and handgrip exercise, the authors independently manipulated the duration of- (i.e. AUC) and the peak shear stress stimulus (with no change in AUC) during reactive hyperaemia. Interestingly, FMD increased in a step-wise manner with increasing AUC, whilst increasing the peak shear stimulus did not affect FMD. These observations suggest that the magnitude of conduit artery vasodilation during reactive hyperaemia is determined by the total post cuff deflation shear stress stimulus, i.e. SRAUC (Pyke & Tschakovsky, 2007).

2.5.3 Cardiovascular Disease Risk Factors

Traditional CVD risk factors include age, sex, ethnicity, smoking, diabetes, blood pressure, body mass index (BMI), cholesterol, family history and physical inactivity. Over the past decades, various CVD risk score calculators have been produced using a combination of the above risk factors to predict future CVD risk. A frequently-used prediction tool is the Framingham risk score. Despite the independent prognostic value of FMD for future CVD events (Ras et al., 2013; Inaba et al., 2010; Matsuzawa et al., 2015), the FMD has failed to add value to traditional CVD risk factors in terms of risk

stratification (Peters et al., 2012). Population-based studies have reported an inverse relation between FMD and Framingham risk score (Yao et al., 2014; Kwagyan et al., 2009; Campuzano et al., 2006). Given the multi-factorial nature of CVD, one may question the relative importance of individual risk factors on FMD. Engelen et al. (2013) established reference values for carotid IMT in a healthy subpopulation and examined the relation between carotid IMT and CVD risk factors. Interestingly, systolic blood pressure, smoking and diabetes were the strongest determinants of a larger carotid IMT compared to healthy individuals of the same age and sex. Evidence for the negative effect of CVD risk factors on FMD are discussed below.

2.5.3.1 Metabolic Syndrome and Diabetes Mellitus

Metabolic syndrome (MetS) is characterised by the presence of a cluster of CVD/metabolic risk factors defined by the National Cholesterol Education Program Adult Treatment Panel III (ATP-III) guidelines (Reilly & Rader, 2003; Eckel et al., 2005). These guidelines classify MetS based on thresholds for waist circumference, blood metabolites (cholesterol, glucose and triglycerides) and blood pressure. Previous studies have shown these risk factors are all independently associated with decreased endothelium-dependent vasodilation (Benjamin et al., 2004; Rodriguez et al., 2005; Kuvin et al., 2003; Brook et al., 2001). Interestingly, Melikian et al. (2008) observed a stepwise decline in FMD with the presence of each additional ATP-III criteria in asymptomatic individuals with and without MetS. Also, individuals with MetS demonstrated significantly greater levels of inflammatory markers (Melikian et al., 2008), implying the pivotal role of NO as the underlying mechanism of this relationship. These results have been confirmed in a large population (Skaug et al., 2014), with emerging sex differences in the effect of MetS risk factors on FMD. Additionally, endothelium-dependent vasodilation is markedly impaired in obese adults with MetS compared with metabolically healthy obese adults (Schinzari et al., 2015). Conversely, Fernandes et al. (2014) observed a non-significant

difference (1%) in FMD. Although not statistically significant, this may be clinically meaningful as a 1% increase in FMD is associated with an 8-13% decrease in CVD risk (Ras et al., 2013; Inaba et al., 2010; Matsuzawa et al., 2015).

Diabetes mellitus (both Type 1 and 2) is associated with endothelium-dependent dysfunction (Avogaro et al., 2006; Calles-Escandon & Cipolla, 2001; Henry et al., 2004; Hamilton & Watts, 2013), whilst some have also observed impaired endothelium-independent function (Williams et al., 1996; Lim et al., 1999; Beckman et al., 2003; Furuta et al., 2013). Furthermore, Type 2 patients have a larger degree of endothelial dysfunction compared to individuals with Type 1 (Ohsugi et al., 2014), which may be driven by the group differences in BMI. Interestingly, Naylor et al. (2011b) observed a step-wise increase in FMD between Type 2, (metabolically healthy) obese and lean adolescents, respectively. This suggests that endothelial dysfunction in Type 2 diabetes, even in youth, is mediated by a metabolic pathway in addition to obesity.

The vascular pathophysiology provoked by cardiometabolic complications occurs at a cellular level, which ultimately negates NO bioavailability. Firstly, impairment in the insulin signalling pathway leads to declined eNOS and NO production (Hamilton & Watts, 2013). Additionally, oxidative stress appears to play a role in endothelial dysfunction within this population (Capellini et al., 2010; Pitocco et al., 2010; Anderson et al., 2005). Observations of elevated adhesion molecule expression (el-Mesallamy et al., 2007), and greater platelet aggregation (Anderson et al., 2005) are present in Type 2 patients, further implying blunted NO bioactivity and development of atherosclerosis. The contribution of each individual MetS risk factor on impaired FMD will be discussed below.

2.5.3.2 Hypertension

Findings from the Framingham Heart study showed an inverse relationship between systolic blood pressure and brachial artery FMD (Benjamin et al., 2004). Comparative studies have demonstrated that hypertensive patients have impaired endothelium-dependent function compared to normotensive individuals (Ghiadoni et al., 2001; Taddei et al., 2001), which was offset by anti-hypertensive treatment (Taddei et al., 2002), implying blood pressure-induced endothelial damage in hypertension. This association is believed to be primarily mediated by oxidative stress. For example, experimental evidence in mice carotid arteries has shown that increased blood pressure is related to the production of oxidative stress (Vecchione et al., 2009) whilst in humans anti-oxidant therapy improved brachial artery FMD in hypertensive patients (Plantinga et al., 2007). Other potential mediating mechanisms include increases in arterial stiffness (Mitchell et al., 2005; Plantinga et al., 2007) and larger artery diameter from vascular remodelling (Chung et al., 2009), which may be induced by the prolonged exposure to hypertension.

2.5.3.3 Obesity

Numerous studies have observed a relationship between body size (i.e. BMI) and artery diameter (Chung et al., 2009; Thijssen et al., 2011b; Parikh et al., 2009; Benjamin et al., 2004; Schroeder et al., 2000), which is a confounding variable on FMD. Furthermore, visceral adiposity, an independent CVD risk factor (Mahabadi et al., 2009), has also been associated with artery diameter and inversely associated with FMD across the lifespan (Hashimoto et al., 1998; Brook et al., 2001; Marchesi et al., 2007; Parikh et al., 2009; Konrad et al., 2011; Tounian et al., 2001; Watts et al., 2004). Importantly, exercise and/or dietary interventions resulting in weight loss, or visceral fat loss without changing weight (Watts et al., 2004), have been successful in improving endothelial function (Pierce et al., 2008; Bigornia et al., 2010; Ades et al., 2011).

Obesity-induced endothelial dysfunction is multifactorial, and largely mediated through impaired NO synthesis and release (Shankar & Steinberg, 2005). It has previously been reported in obesity that endothelial cell adhesion molecules are increased (Escobar-Morreale et al., 2003), implying a decline in NO function given the protective properties described in *Section 2.1.2*. Increased inflammatory markers (Escobar-Morreale et al., 2003) may impair NO production (Steinberg et al., 1997) and induce oxidative stress (Shankar & Steinberg, 2005). Furthermore, elevated free fatty acids may be related to increased levels of endogenous eNOS inhibitor, asymmetric dimethyl-arginine (Lundman et al., 2001; Stuhlinger et al., 2002), which is associated with hypercholesterolaemia and Type 2 diabetes (Sibal et al., 2010) and inversely associated with endothelial function (Juonala et al., 2007).

2.5.3.4 Dyslipidaemia

Cholesterol levels, comprised of LDL and HDL cholesterol also influence vascular function. More specifically, a combination of high LDL and low HDL is associated with endothelial dysfunction (Celermajer et al., 1994a; Clarkson et al., 1996; Kuvin et al., 2002; Lupattelli et al., 2003; Lupattelli et al., 2002) and subsequent CVD risk (Abbott et al., 1988; Miller et al., 1977). Interestingly, Mullen et al. (2001) reported a lower brachial artery FMD in patients with hypercholesterolaemia compared to healthy controls despite exhibiting a similar SRAUC, implying reduced NO bioavailability/endothelial cell sensitivity. Furthermore, in a cohort of 1583 subjects, hypercholesterolaemia and obesity were associated with a larger artery diameter (Chung et al., 2009), which in turn is inversely associated with FMD (Thijssen et al., 2008a).

In vitro evidence suggests that high HDL promotes eNOS expression (Noor et al., 2007; Ramet et al., 2003), whilst *in vivo*, low HDL was associated with an impaired FMD, but

similar GTN responses (Higashi et al., 2010). This evidence implies an interaction between HDL and the endothelium. Similar to NO, HDL possesses anti-atherosclerotic properties including inhibition of inflammation, platelet adhesion, LDL oxidation and endothelial cell apoptosis (Ansell et al., 2005). Overall, this implies that HDL plays a protective role within the vasculature by promoting NO bioavailability for the maintenance of vascular health and function.

2.5.3.6 Smoking

Cigarette smoking is considered one of the major modifiable risk factors associated with endothelial dysfunction and CVD (Lakier, 1992). A detrimental effect of smoking on FMD has been well established (Wiesmann et al., 2004; Celermajer et al., 1993; Celermajer et al., 1994a; Benjamin et al., 2004; Esen et al., 2004; Yufu et al., 2009; Ozaki et al., 2010; Amato et al., 2013), with no difference in GTN responses or baseline artery diameter. These data imply a direct interaction between smoking by-products and the endothelium. The mediating mechanisms behind the adverse vascular effects of chronic cigarette smoking are complex; nonetheless, a key contributor relates to NO, partially determined by oxidative stress (Mayhan & Sharpe, 1998; Raitakari et al., 2000). Indeed, cigarette smoke contains pro-oxidants and free radicals (Church & Pryor, 1985). Importantly, the negative effects associated with smoking may be partially reversed post-cessation. Early FMD research by Celermajer et al. (1993) observed a greater FMD in ex-smokers compared to current smokers, which has been supported by others on a larger population scale (Johnson et al., 2010). Nonetheless, inconsistent results have been observed with anti-oxidant therapy as a strategy to reverse vascular dysfunction (Puranik & Celermajer, 2003), further demonstrating the complexity of the association between smoking and endothelial function at a cellular level.

2.5.3.7 Ethnicity

Epidemiological research has demonstrated that African Americans have greater CVD risk compared to whites (Mensah et al., 2005). Ample evidence reports ethnic differences in brachial artery FMD (Perregaux et al., 2000; Loehr et al., 2004; Melikian et al., 2007; Yeboah et al., 2008; Shantsila et al., 2011), with Caucasian subjects demonstrating greater FMD compared to African Americans, Blacks, African Caribbean and South Asians. Conversely, some research groups have reported no difference between ethnic groups in FMD response (Gokce et al., 2001; Shantsila et al., 2011; Pusalavidyasagar et al., 2016). However, these results must be interpreted with caution due to heterogeneity in methodology compared to contemporary FMD research following expert-consensus guidelines (Thijssen et al., 2011a). Furthermore, ethnic differences related to vascular health are multi-factorial, with differences in diet and lifestyle mediating this relationship, discussed elsewhere (Pusalavidyasagar et al., 2016).

Experimental evidence *in vivo* and *in vitro* has shown that ethnic differences in endothelial function are determined, at least in part, by oxidative stress (Kalinowski et al., 2004; Feairheller et al., 2011), inflammation (Albert et al., 2004; Brown et al., 2011) and subsequent blunted NO bioavailability. This evidence may explain the predisposition of African Americans to greater CVD risk. In addition, healthy black Africans have been found to have greater plasma asymmetric dimethyl-arginine compared to white Europeans (Melikian et al., 2007), which is negatively associated with endothelial function (Juonala et al., 2007). Whilst ethnicity appears to play a role in vascular dysfunction, existing research thus far possess discrepancies in the FMD methodology and analysis, which limits comparability between studies and the ability to draw definitive conclusions.

2.5.3.8 Physical Activity/Exercise

Current government recommendations state that adults should engage in 150 minutes of moderate intensity or 75 minutes of vigorous intensity physical activity per week as a minimum to maintain health (World Health Organisation, 2010). The cardiovascular health benefits of engaging in physical activity are profound, and there is a curvilinear dose-response relationship between physical activity and disease risk (Department of Health, 2004). According to a recent report, ~39% of adults in the UK fail to meet these guidelines (British Heart Foundation, 2017). It is well documented that physical activity and subsequent greater cardiorespiratory fitness are associated with enhanced endothelial function (Hagg et al., 2005; Davison et al., 2010; Siasos et al., 2013), mediated by the exercise-induced improvement in CVD risk factors and exposure to increases in cyclic blood flow and shear stress (Green et al., 2017; Green & Smith, 2017). However, the effects of physical activity on traditional risk factors only accounted for 59% reduction in CVD risk (Mora et al., 2007), presenting a “risk factor gap” (Green et al., 2008). Therefore, the inclusion of emerging/novel risk factors including cardiorespiratory fitness (DeFina et al., 2013), vascular structure and function (Andersson et al., 2015; Green et al., 2003; Siasos et al., 2013; Kozakova et al., 2010) and autonomic function (Joyner & Green, 2009) may account for the “gap” in CVD reduction achieved through physical activity.

The effects of exercise training on vascular function has recently been reviewed (Green et al., 2017; Green & Smith, 2017). Based on a large number of studies, it became apparent that exercise training can improve conduit artery endothelium-dependent function (Birk et al., 2012; Green et al., 2004; Thijssen et al., 2010) and (in most cases) lead to outward structural remodelling (Green et al., 2004; Dinenno et al., 2001; Spence et al., 2013). These adaptations are most likely provoked by repetitive increases in

transmural pressure, circumferential wall strain and shear stress (Green & Smith, 2017). Shear stress as the stimulus for dilation and adaptation will be discussed below.

2.6 Shear Stress

At the end of the 19th century, it was observed in chick embryos that more branches developed in blood vessels where the blood flow velocity was highest (Thoma, 1893). Whilst this suggests a role for blood flow (or shear stress) in angiogenesis, only a few decades ago, the importance of the endothelium for mediating vascular function and remodelling was observed (Pohl et al., 1986; Berdeaux et al., 1994; Langille, 1996). Animal research demonstrated that an acute elevation in shear stress results in increased production of the enzyme eNOS mRNA, NO and prostacyclin (Malek et al., 1999), whilst chronic exposure to elevated shear stress caused outward structural remodelling (Ben Driss et al., 1997). Since these observations, the endothelium has been a focus for research, given its strategic placement and crucial role in the progression and development of atherosclerosis (McLenachan et al., 1991). Shear stress plays a pivotal role in the development and progression of CVD (Cecchi et al., 2011) due to the direct interaction with the endothelium (i.e. mechanotransduction (Chien, 2007)). It is now widely accepted that vascular adaptation, both relevant for acute changes in diameter as well as chronic adaptations in remodelling, is dependent upon an intact, functional endothelium (Ross et al., 1984; Zeiher et al., 1989). Furthermore, in addition to an intact endothelium and structural properties (i.e. wall thickness and stiffness), other factors must be considered in determining the magnitude of FMD. These include the amount and pattern of shear stress, transduction of the dilatory response to the vascular smooth muscle cells, and the response of the vascular smooth muscle cells to dilator signals via changes in calcium levels (Koller & Kaley, 1991; Lehoux et al., 2006; Thijssen et al., 2008a).

2.6.1 Stimulus for Adaptation

Shear stress plays an important role in inducing functional and structural adaptations, facilitated by the endothelium. Increasing shear stress through heat exposure mediates acute (Tinken et al., 2009; Greyling et al., 2015a) and chronic (Naylor et al., 2011a) elevation in vasodilator function. Tinken et al. (2009) demonstrated shear stress-induced improvements in brachial artery FMD immediately following acute forearm heating, cycling and handgrip exercise. Furthermore, preventing the increase in antegrade shear stress during these interventions with low levels of unilateral pneumatic cuff inflation abolished this effect. To follow-up on these observations of the acute impact of exercise, studies have examined the impact of repeated exposure to these stimuli. For example, an 8-week treadmill exercise training protocol resulted in an increase in brachial artery FMD at weeks 2, 4 and 6, which subsequently returned to baseline levels at week 8 (Tinken et al., 2008). Interestingly, these functional adaptations were superseded by structural remodelling, which explains the functional return to baseline levels (Figure 2.7). This confirms the hypothesis proposed by Laughlin (1995) regarding the time course of change in vascular function and structure with exercise training-mediated increases in shear stress. To directly examine the role of shear stress in exercise training-induced functional adaptations, FMD was assessed at 2-week intervals during an 8-week bilateral handgrip exercise with unilateral cuff inflation protocol (Tinken et al., 2010). Similar to previous observations, FMD increased at weeks 2, 4 and 6 in the non-cuffed arm and returned to baseline levels at week 8 (with no change in the cuffed arm). Therefore, reducing the antegrade component of the shear stress pattern with low levels of unilateral pneumatic cuff inflation abolished the increased FMD (Tinken et al., 2010), further emphasising the importance of shear stress in vascular adaptation to exercise.

The adaptations during handgrip or cycling exercise training may not only be caused by shear stress *per se*, since exercise is associated with a multitude of cardiovascular health

outcomes. For this reason, previous work has examined whether repeated increases in shear stress mediated through heat exposure can lead to adaptation. The sustained increase in antegrade shear stress observed during heating is associated with immediate improvement in endothelium-dependent function (Naylor et al., 2011a; Brunt et al., 2016; Carter et al., 2014). Importantly, this method to improve vascular health through heat emphasises the importance of elevation in mean shear stress to mediate vascular adaptations, but also may serve as a simple and novel strategy to improve vascular function in clinical populations, i.e. with limited mobility and/or unable to exercise.

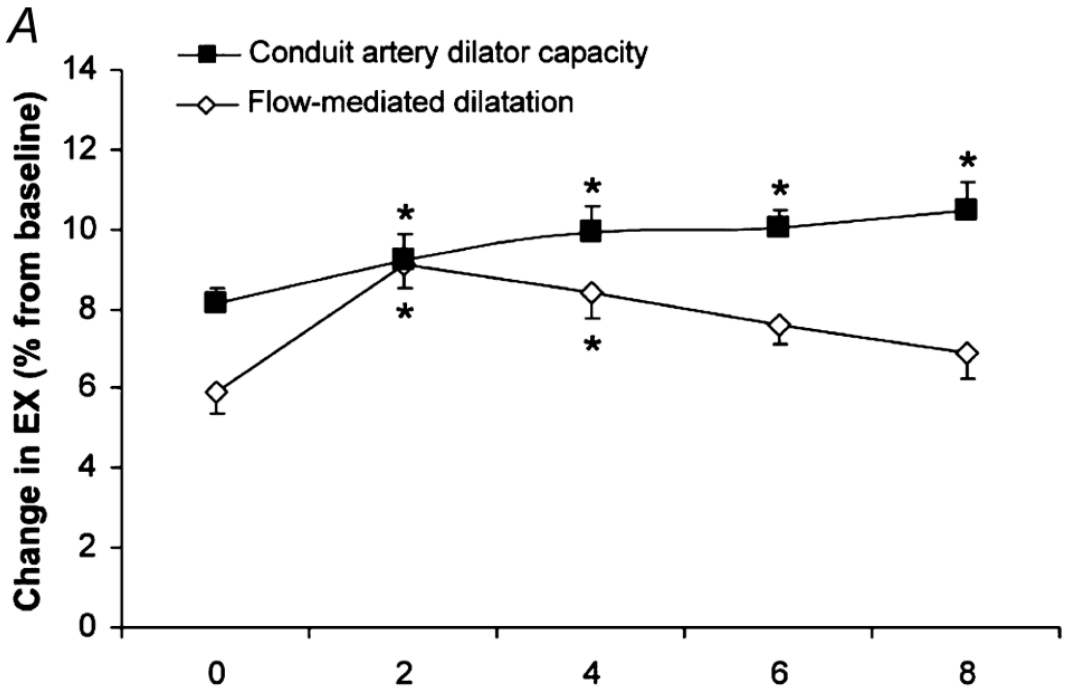

Figure 2.7: Brachial artery FMD and dilatory capacity during an 8-week exercise training intervention, from Tinken et al. (2008).

2.6.2 Shear Patterns

Shear stress has two main components; antegrade (shear towards to periphery) and retrograde (shear towards the heart). The relevance of the pattern of shear stress has been demonstrated *in vivo* and with the use of *ex vivo* simulation of blood flow and wall shear stress (Steinman et al., 2002; Peiffer et al., 2013; Davies et al., 2010). More

specifically, arterial wall thickening is more likely to occur at bifurcations where wall shear stress is multi-directional, suggesting that shear pattern may be an influencing factor in atherogenesis (Steinman et al., 2002; Peiffer et al., 2013; Davies et al., 2010). Furthermore, experimental studies have shown that increasing the antegrade or retrograde shear component is associated with enhanced or impaired vasodilator function, respectively (Thijssen et al., 2009c; Schreuder et al., 2015; Johnson et al., 2012). The importance of shear patterns has been explored *in vivo* using handgrip exercise to produce fluctuations in shear stress. Pyke et al. (2008) compared the vasodilation response to the same mean shear stress stimulus with different shear pattern (i.e. fluctuations around the mean *versus* stable). Interestingly, the authors found no difference in the magnitude of vasodilation, implying that the endothelium is sensitive to mean shear stress levels. These observations were supported by a later study of handgrip exercise showing no difference in FMD between conditions imposing varying magnitude of fluctuations with preserved mean shear stress levels (King et al., 2013). However in the above studies, mean shear was markedly different from resting baseline levels, and exposure to prolonged periods of continuous elevations in antegrade or retrograde shear stress do not happen regularly in daily living.

Recently, a research group in Norway developed and utilised a method of intermittent negative pressure (10 seconds negative pressure (-40mmHg), 7 seconds atmospheric pressure) to induce fluctuations in blood flow and shear stress with no changes in central hemodynamics (Sundby et al., 2016b). This equipment comprises of a custom-made boot-shaped plastic vacuum chamber, which is sealed around the knee, connected to a pressure control system pre-programmed for the 17-second cycles (Figure 2.8). The authors observed a significant increase in blood flow and shear rate during the application of negative pressure, whilst flow and shear rate dropped to below baseline levels during atmospheric pressure (Figure 2.8). This induced fluctuations in shear stress

levels without altering mean shear stress. Interestingly, Sundby and colleagues conducted a series of elegant experiments in patient groups with peripheral arterial disease and spinal cord injury (Sundby et al., 2018a; Sundby et al., 2016a; Sundby et al., 2017; Sundby et al., 2018b), and the findings from these studies demonstrated that the fluctuations in blood flow facilitated lower limb wound/ulcer healing. The authors speculated that these positive clinical outcomes could be attributed to shear stress-induced improvements in endothelial function. However, this is currently unknown, therefore further research is warranted to explore the mechanisms involved in the use of intermittent negative pressure. Insight into this area will both be relevant to understand the clinical benefits of fluctuations in shear stress, but also highlight the physiological relevance of exposure to fluctuations in shear stress with mean shear stress levels being kept relatively stable.

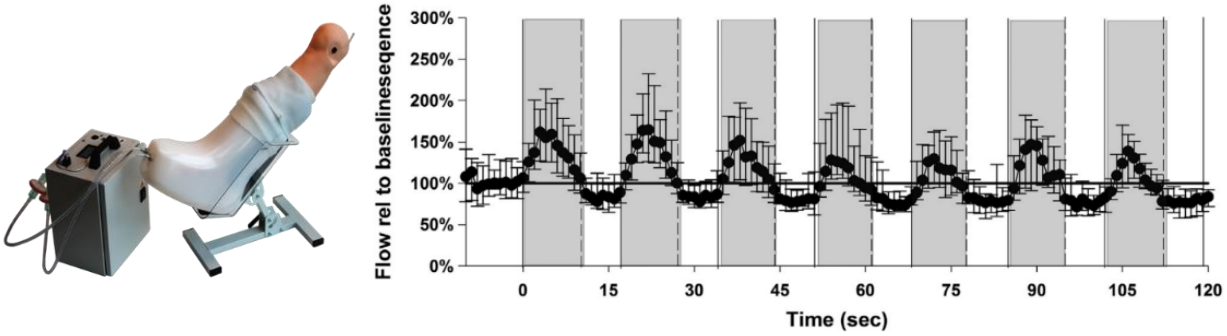

Figure 2.8: Experimental set-up of the negative pressure system (left), and the fluctuations in blood flow observed during intermittent negative pressure (right), from Sundby et al. (2016b).

2.7 Summary

In summary, whilst CVD remains the world's leading cause of death, most CVDs are preventable which highlights the importance of early detection. Given that endothelial dysfunction is an early biomarker of CVD and is present before an atherosclerotic plaque is visible, the FMD may represent a valuable research tool in predicting and preventing CVD. Nonetheless, important questions remain unanswered. First, since adherence to

FMD protocol guidelines importantly improve reproducibility, an update of the 2011 guidelines seem warranted to improve technical performance of the FMD. Secondly, reference vales for the FMD are yet to be established. Third, the role of shear stress as a hemodynamic stimulus for vasodilation is well recognised, but the exact impact of age and sex on the relationship between FMD and the eliciting shear stimulus is unclear. Finally, the impact of exposure to constant levels of an increase or decrease in shear on FMD is well established. However, emerging evidence is suggestive of positive health-related outcomes associated with fluctuations in shear stress.

Chapter 3

Evolution of the FMD Test and Implications for CVD Risk Prediction

Based on published review (see appendix for full article):

Thijssen, D.H.J., Bruno, R.M., Van Mil, A., **Holder, S.M.**, Faita, F., Greyling, A., Zock, P., Taddei, S., Deanfield, J.E., Luscher, T., Green, D.J., & Ghiadoni, L. (2019). *Expert consensus and evidence-based guidelines for the assessment of flow-mediated dilation (FMD) in humans.* European Heart Journal, 40(30), 2534-2547.

3.1 Background

The FMD test was introduced in 1992 when it was first utilised by Celermajer et al. (1992) to assess changes in artery diameter in response to increased flow. This first study was performed in children and adults at risk of atherosclerosis compared to healthy controls. Following a period of rest, blood flow through the brachial artery was restricted with a pneumatic tourniquet inflated to a suprasystolic pressure, and releasing this pressure induced a reactive hyperaemic response and subsequent vasodilation. Interestingly, the authors observed significant differences in brachial artery dilation between healthy individuals, smokers and coronary artery disease patients, where the magnitude of dilation declined in a dose-dependent manner. Given the nature of the methodology, this technique became known as the FMD. Since then, several research groups worldwide have adopted this technique to assess conduit artery endothelial function. Over the years, however, this technique has been modified to improve reproducibility and reliability, with methodological guidelines being introduced by Corretti et al. (2002) and updated by Thijssen et al. (2011a).

The current brachial artery FMD test protocol consists of one minute of baseline brachial artery diameter and blood flow velocity recording, followed by a 5-minute period of distal limb ischaemia, induced by inflating a pneumatic cuff to suprasystolic level (~220mmHg), placed distal to the humeral epicondyle. Following cuff release, artery diameter and blood flow velocity are recorded for a further 3 minutes (Thijssen et al., 2011a). Subsequently, FMD is calculated as the relative (percentage) change in diameter from baseline to peak following cuff release. This chapter will review studies within the area of physiology which contributed to the development of expert-consensus guidelines (latest guidelines published in *European Heart Journal*).

3.2 Protocol: Occlusion Cuff Position and Duration

The effect of cuff position (distal *versus* proximal to the imaging site) has previously been investigated experimentally (Doshi et al., 2001; Betik et al., 2004). Importantly, changes in cuff placement can alter the magnitude (Naylor et al., 2005a), duration (Doshi et al., 2001; Naylor et al., 2005a), nature (Green et al., 2014) and even the clinical relevance (Green et al., 2011) of the FMD response. Below, these aspects have been discussed in relation to the position of the occlusion cuff.

3.2.1 Distal Versus Proximal Cuff Position

In the original paper by Celermajer et al. (1992), brachial artery FMD was examined with the occlusion cuff placed distal around the forearm, ~1 cm below the elbow crease (Figure 3.1). However, some researchers have supported the placement of the occlusion cuff proximal to the imaged artery (Doshi et al., 2001). Multiple research groups have demonstrated that proximal cuff occlusion causes a larger FMD response compared to distal occlusion (Berry et al., 2000; Agewall et al., 2001; Betik et al., 2004; Doshi et al., 2001), and this is partially due to a greater shear stimulus elicited during reactive hyperaemia (Green et al., 2011). From this, it has been argued that adopting the proximal cuff method may improve the ability to detect subtle differences between populations or within subjects. Nonetheless, a previous analysis found no significant differences in prognostic value between studies that adopted distal *versus* proximal cuff position (Green et al., 2011). Furthermore, evidence suggests that the dilatory response following cuff release may be mediated by other vasodilators than NO alone. A recent meta-analysis by Green et al. (2014) investigated the degree to which FMD is mediated by NO. Out of the 20 studies included in the meta-analysis, four used proximal cuff placement, whilst the remaining research groups adopted the traditional distal cuff method. The authors evaluated the potential effects of the difference in cuff position and the subsequent importance of distal cuff occlusion was highlighted, whereby ~72% of

conduit artery vasodilation was NO-mediated when the occlusion cuff was placed distal to the imaging site. However, when studies adopting proximal cuff placement were included in the analysis, FMD was only ~32% NO-mediated.

The between-study differences in cuff position makes comparisons in FMD data difficult, and this is of clinical importance since several studies specifically aim to study the NO-pathway given its role in the atherosclerotic process. Thus, strict standardisation of cuff occlusion below the artery is now recommended to ensure maximal dependence of the FMD response on endothelium-derived NO.

3.2.2 Duration and Magnitude of Cuff Occlusion

In addition to cuff position, the duration of cuff occlusion is also important and has a direct impact on the magnitude of the dilatory response (Leeson et al., 1997; Mullen et al., 2001; Naylor et al., 2005a). Leeson et al. (1997) assessed FMD in response to graded cuff occlusion duration (30 seconds, 1.5-, 2.5-, 3.5-, 4.5- and 8 minutes). Interestingly, the authors observed a stepwise increase in FMD with increasing occlusion duration, whilst a plateau was observed between 4.5-8 minutes. Therefore, current guidelines recommend adopting the 5-minute cuff occlusion period. Furthermore, the pressure of the cuff during occlusion should be at least 50 mmHg above the participant's systolic blood pressure to prevent arterial inflow.

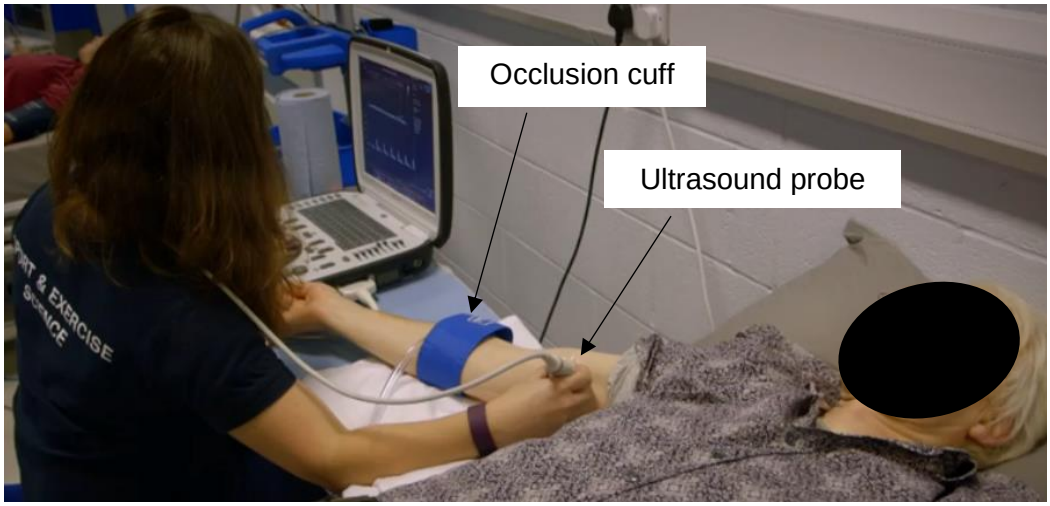

Figure 3.1: Set-up for the brachial artery FMD protocol, with the occlusion cuff placed distal to the imaging site.

3.3 Protocol: Examining the Time Course of Peak Artery Diameter

Original studies adopting the FMD approach assessed peak diameter using single B-mode frames at 60 seconds post cuff release (Celermajer et al., 1992; Celermajer et al., 1994a) and this time frame was doubled in the 2002 guidelines (Corretti et al., 2002). However, experimental evidence has shown that this approach (including pre-determined time windows) can result in an underestimation of true FMD. Black et al. (2008) measured FMD in 36 subjects, equally split into young, trained old and sedentary old groups. The authors used various time windows to assess peak diameter, including the traditional 60 seconds, 50-70-, 70-90-, 0-90- and 0-120 seconds. In all groups, the peak diameter at 60 seconds was significantly lower than the true peak FMD. Interestingly, the majority of subjects (34 out of 36) reached their peak diameter within the first 2 minutes of reactive hyperaemia, with the remaining two belonging to the sedentary old group. This emphasises that timing of peak dilation differs between groups, and may alter following interventions such as exercise training (Thijssen et al., 2011c; Liuni et al., 2010). Therefore, to ensure successful capture of the true peak diameter, current guidelines support examining up to 3 minutes post cuff release in the brachial artery.

3.4 Analysis: Identifying Peak Artery Diameter

Initial FMD studies used manual analysis techniques to examine peak artery diameter, where investigators visually inspected a single frame of the B-mode image 60 seconds post cuff release and applied calipers along the artery diameter (Celermajer et al., 1994a; Celermajer et al., 1992). Later, the 2002 FMD guidelines by Corretti et al. (2002) recommended identifying the peak diameter during FMD using ECG-gated analysis. This method assesses the artery diameter at the onset of the R wave (end diastole, when the artery diameter is smallest) of every cardiac cycle, in order to limit the influence of arterial compliance. These manual analysis methods are highly operator-dependent and therefore subject to observer error and inaccurate FMD data (Woodman et al., 2001; Williamson et al., 2008; Harris et al., 2010; Mancini et al., 2002; Preik et al., 2000). Nonetheless, these methods have now been superseded by continuous diameter assessment, which calculates an average diameter across the cardiac cycle to determine baseline and peak artery diameter (Figure 3.2). Continuous diameter assessment has been found to have strong agreement in FMD and GTN data with ECG-gated analysis (Gemignani et al., 2008; Kizhakekuttu et al., 2010) and is also more time efficient (Kizhakekuttu et al., 2010). Therefore, given the accuracy and availability of continuous assessment systems, ECG gating should no longer be mandatory to determine peak artery diameter during FMD. The importance of continuous artery diameter assessment will be discussed below.

3.4.1 Edge-Detection and Wall-Tracking

Given how small the total artery diameter change is (0.1-0.4mm) and observer error associated with manual analysis methods, automated edge-detection and wall-tracking analysis methods demonstrate greater validity and reproducibility compared to manual analysis (Woodman et al., 2001; Williamson et al., 2008; Mancini et al., 2002; Preik et al., 2000; Sonka et al., 2002). A comparison study found a considerable difference in

FMD output between automated *versus* manual edge-detection, where all parameters of endothelium-dependent and -independent FMD, including baseline diameter, total shear rate and percentage change were significantly under-estimated by the manual method compared to the automated system (Williamson et al., 2008). This complicates the comparability of FMD data between studies using different analysis techniques. The manual technique carries significantly greater observer bias and intra-observer variation (Woodman et al., 2001), and although whilst using edge-detection software, the initial selection of the region of interest is operator-dependent based on image quality, all subsequent analysis is observer-independent and carried out without investigator bias (Green & Reed, 2006). Edge-detection and wall-tracking software uses pixel density and frequency distribution algorithm to assess B-mode frames (Figure 3.3), where the artery walls and blood flow velocity trace are automatically detected. Furthermore, these softwares automatically calculate FMD and SRAUC (from cuff release to peak diameter; highlighted in red in Figure 3.2). Therefore, given the current availability of several edge-detection and wall-tracking analysis systems and its superiority in terms of validity and bias, validated, accurate and reproducible edge-detection and wall tracking systems should be used where possible.

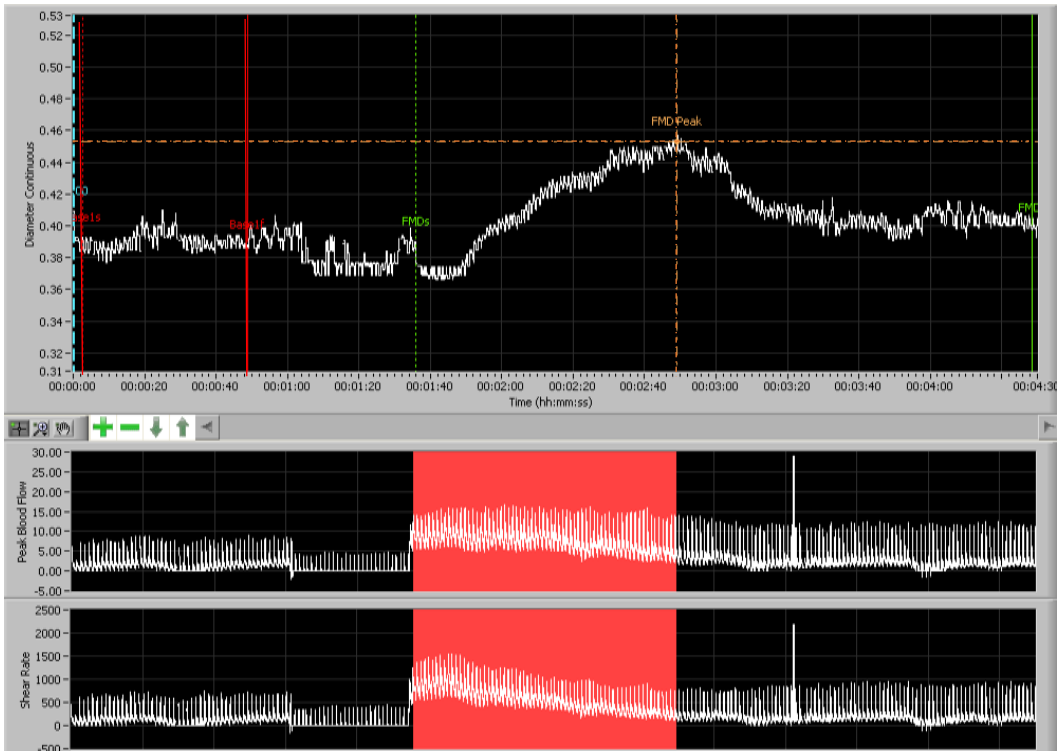

Figure 3.2: Screenshot of FMD analysis, including artery diameter (**top**), peak blood flow (**middle**) and shear rate (**bottom**). Baseline diameter is calculated as the average diameter between the two red bars, and the peak is determined automatically by the software (shown by the orange dashed lines; **top panel**).

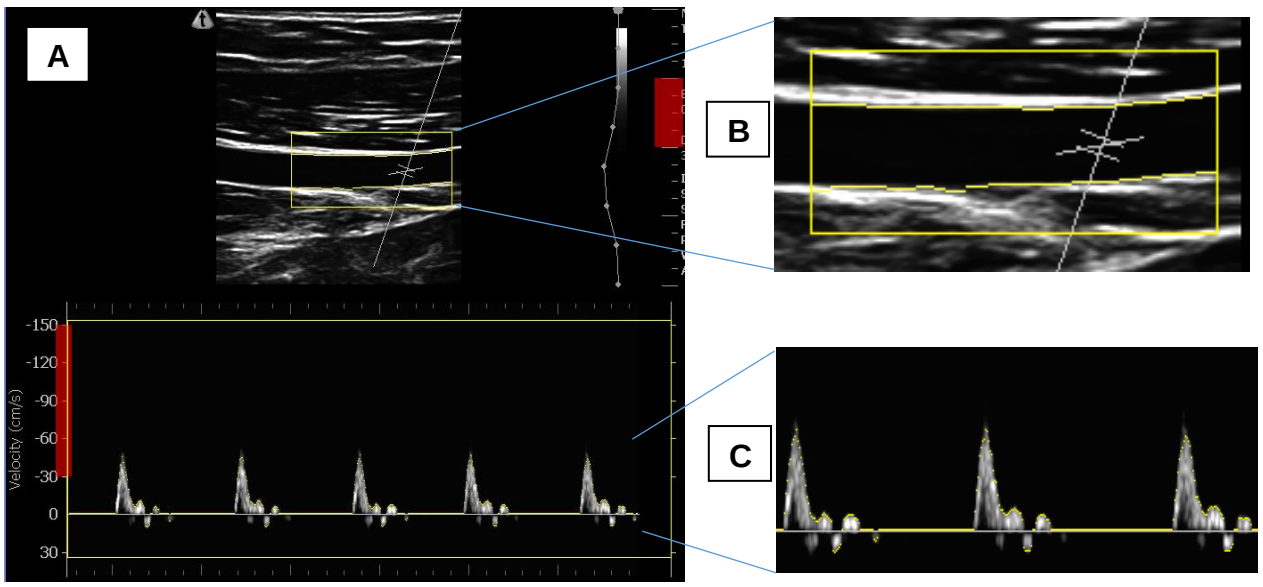

Figure 3.3: A) Screenshot of edge detection software (diameter and velocity), **B)** Region of interest selected by the observer on a section of the artery and software tracking the artery walls, **C)** Edge-detection software tracking the blood flow velocity trace.

3.5 Normalisation for FMD Stimulus

Based on the importance of the shear stress stimulus during reactive hyperaemia (Pyke & Tschakovsky, 2007), as well as the mathematical and physiological effect of baseline diameter on the FMD (Thijssen et al., 2008a), it was suggested that these variables should be statistically controlled for during subsequent data analysis. Various approaches have been put forward by statisticians to independently control for shear rate and baseline diameter, and these will be discussed below.

3.5.1 Shear Stress Stimulus

Given the between- and within-group variability of SRAUC (Padilla et al., 2008; Pyke et al., 2004; Ishibashi et al., 2006), it may be unclear whether these differences are attributable to biological variability in endothelial function or differences in the magnitude of the shear stimulus. This raised the question of how to account for the shear stimulus when interpreting FMD. Researchers began normalising FMD responses by dividing the FMD by shear rate to produce a ratio (de Groot et al., 2004; Parker et al., 2006a; Padilla et al., 2008). However, this type of 'simple' ratio normalisation is statistically flawed as it fails to meet important statistical assumptions; (i) the relationship between both parameters is linear, (ii) the intercept for the regression slope of this relationship is zero, (iii) data (including residuals) are normally distributed, (iv) variances are similar between groups and (v) the ratio does not lead to spurious correlations with other variables (Atkinson et al., 2009). Atkinson et al. (2009) found that all assumptions for the reliable use of FMD/shear rate ratios were violated in the comparison of FMD between boys, young men and older men. This evidence suggests that ratio normalisation does not work as it cannot simply be corrected for.

Further exploration into statistical strategies to correct for the shear stimulus has investigated the use of SRAUC as a covariate in an analysis of covariance (Harris & Padilla, 2007; Atkinson et al., 2009; Atkinson & Batterham, 2013b). However, these results could be misleading if the covariate, i.e., shear rate, is related to the independent- and outcome variable (for example, age, sex, baseline diameter and FMD). Further investigation is required to determine the potential utility of this method for normalisation in the future, though it seems appropriate for repeated FMD measurement, but not for cardiovascular risk prediction.

3.5.2 Baseline Diameter

The mathematical influence of baseline diameter partly contributes to the known inverse relationship between baseline diameter and FMD (Thijssen et al., 2008a). Allometric scaling was proposed to adjust for the influence of baseline diameter on FMD (Atkinson & Batterham, 2015; Atkinson & Batterham, 2013b). A study by Atkinson (2014) re-analysed 44 pairs of FMD/shear normalisation data from Pyke et al. (2004) using the allometric approach and the new re-analysis revealed that baseline diameter-dependency could be eliminated. However, whilst allometric scaling may have physiological relevance, it is unclear what this adds to the current prognostic value.

3.5.3 Normalisation for FMD Stimulus: Summary

In summary, no clear statistical strategy is currently in place to account for the eliciting shear rate stimulus during FMD. Ratio normalisation appears statistically flawed, whilst too many questions remain around validity, feasibility and interpretation of results as an index of endothelial function and cardiovascular risk. Guidelines recommend reporting the relevant shear rate stimulus, either as the peak and/or AUC. Reporting this data may also be clinically relevant since previous work found that the shear stimulus during

reactive hyperaemia may offer prognostic value, given its association with cardiovascular risk in healthy and diseased populations (Mitchell et al., 2004; Philpott et al., 2009; Huang et al., 2007; Paine et al., 2016).

3.6 Reproducibility of FMD

A nationwide multi-centre study assessed the effect of standardised methodology on the reproducibility of the FMD (Ghiadoni et al., 2012). The FMD test was conducted on 135 healthy participants at baseline, one hour later and 30 days later. Importantly, the seven centres involved in the study strictly followed expert-consensus guidelines regarding the test protocol and edge-detection analysis system (Thijssen et al., 2011a) and the sonographers had also received optimal training. Reproducibility is reported as a coefficient of variation (calculated as (standard deviation/mean)x100) and current guidelines indicate that FMD coefficient of variation should be below 20–30% (Corretti et al., 2002). The results of the multi-centre study demonstrated a coefficient of variation of 11.6-16.1%, which demonstrates that by following the guidelines and with sufficient technical training, FMD is highly reproducible.

Recently, a scoring tool was designed to assess the level of guideline adherence for the FMD, specifically related to subject preparation, image acquisition and analysis (Greyling et al., 2016b). Using this tool, the authors assessed the relationship between adherence score and reproducibility of the FMD between studies (Greyling et al., 2016a) and found that strict guideline adherence markedly improved the reproducibility of the brachial artery FMD. Together, the above research highlights the importance of adhering to expert-consensus guidelines for highly reproducible and reliable results.

3.7 Summary and Conclusion

The FMD has proven valuable in providing a non-invasive assessment for future CVD risk, even in asymptomatic individuals (Inaba et al., 2010; Ras et al., 2013; Green et al., 2014). However, a major limitation of the current available literature is that there is varying level of adherence to expert-consensus guidelines, which limits the comparability and validity of FMD results between studies and laboratories. Given the importance of following these guidelines on reproducibility and reliability, as discussed above, research groups are encouraged to perform the FMD in accordance with expert-consensus guidelines. The full article and recommendations can be found in the appendix. Implementing standardised methodology adds to the prognostic value of the FMD for future CVD, but will also contribute to the development of reference values. Importantly, the construction of reference values would allow the FMD test to be applicable in a clinical setting rather than just used as a research tool. However, to date, no previous study has attempted to construct FMD reference values.

Chapter 4

Age- and Sex-Specific Reference Values for Brachial Artery FMD and the Relation with CVD Risk Factors

4.1 Introduction

Since its introduction, FMD has become a popular research tool used worldwide to assess endothelial function. Nonetheless, despite the independent prognostic value of FMD (Ras et al., 2013; Xu et al., 2014; Inaba et al., 2010; Matsuzawa et al., 2015), some limitations hamper widespread use of the technique. As reviewed in *Chapter 2*, age- and sex-specific differences in FMD have been consistently reported (Seals et al., 2011; Hopkins et al., 2015; Adams et al., 1996; Celermajer et al., 1994b; Taddei et al., 1995; Yao et al., 2014) with older age and male sex being associated with lower FMD values. However, marked differences in FMD values are present between studies, prohibiting meaningful comparisons. These differences are largely attributed to variation in FMD methodology between studies (e.g. timing, occlusion cuff position, and manual *versus* automated analysis). Therefore, consistent implementation of expert-consensus guidelines (Thijssen et al., 2011a; Thijssen et al., 2019b) seems a logical solution to lower FMD variability (Greyling et al., 2016a). Moreover, studies have not controlled for age-related changes in diameter and CVD risk factors, i.e. (patho)physiological indices that importantly contribute to the magnitude and variation of the FMD. This highlights the need and importance of age- and sex-specific reference values for FMD, collected when adhering to protocol guidelines, to facilitate interpretation of FMD outcomes.

For this study, FMD observations from six laboratories that all strictly adhered to protocol and analysis guidelines (Corretti et al., 2002; Thijssen et al., 2011a) were pooled into a single database. First, using data from 1,403 healthy individuals, age- and sex-specific reference intervals for brachial artery FMD across the entire lifespan were established. This data also facilitated exploration into the role of age- and sex-specific differences in artery diameter on FMD. Secondly, 3,959 individuals with established CVD risk factors (i.e. above international cut-off normative values) were added to the dataset, to explore

how these risk factors, as well as medication use, impacted the age- and sex-specific FMD reference values.

4.2 Methods

4.2.1 Participants

Research groups from the International Working Group on Flow-Mediated Dilation identified eligible studies that included assessment of brachial artery FMD. Studies were included if all measurements were performed with adherence to the expert-consensus guidelines on measuring FMD (Corretti et al., 2002; Thijssen et al., 2011a) and data collection adhered to the Declaration of Helsinki.

Individual-level brachial artery FMD data with corresponding participant characteristics and medical history from six laboratories were compiled (for the list of contributing laboratories and investigators, see appendix). With permission from principal investigators, unpublished data was included (32% of total observations). When the original studies adopted a methodological design with repeated FMD measurements, the first-performed FMD was included.

For the first objective, i.e. age- and sex-specific reference intervals, healthy individuals (4-84 years; 821 males and 582 females) were selected following stringent inclusion criteria (Mancia et al., 2013) including (when available): (i) systolic blood pressure <140mmHg and diastolic blood pressure <90mmHg, (ii) BMI <25kg/m^2, (iii) waist circumference <102cm for males and <88cm for females, (iv) total cholesterol <4.9mmol/L, (v) LDL <3mmol/L, (vi) HDL >1mmol/L for males and >1.2mmol/L for females, (vii) triglycerides <1.7mmol/L, (viii) glucose <5.6mmol/L, (ix) never smoked, (x)

no history of metabolic- or CVD/event, and (xi) not taking any medications or hormone-based contraception/therapy. For the second objective, i.e. impact of CVD risk factors (BMI, blood pressure, cholesterol, diabetes, dyslipidaemia and smoking) , the remaining participants with one or more risk factor (n=3,959) were stratified into un-medicated (males; n=545, females; n=247), and medicated individuals (males; n=1920, females; n=1247). The medicated subpopulation were taking blood pressure-, lipid- and/or glucose-lowering drugs.

4.2.2 Brachial Artery FMD: Methodological Considerations

Importantly, only brachial artery FMD data from research groups strictly adhering to expert-consensus guidelines (Corretti et al., 2002; Thijssen et al., 2011a) were included in the dataset. FMD assessments were performed following standardised participant preparation procedures (i.e. fasted state, abstained from exercise, caffeine and alcohol, and timing of menstrual cycle). Following 10-15 minutes of supine rest, brachial artery diameter was assessed via high-resolution duplex ultrasound using a hand-held probe or probe-holder approach. B-mode images were obtained and optimized, and Doppler velocity was recorded simultaneously. After at least 1 minute of baseline diameter and blood flow velocity measurement, an occlusion cuff, placed distal to the olecranon process, was inflated to suprasystolic pressure (i.e. >50mmHg above the participant's systolic blood pressure) for 5 minutes. Recordings were resumed 30 seconds before cuff deflation, and FMD was recorded for a further 3 minutes post cuff deflation.

FMD data were analysed using automated edge-detection and wall-tracking software (BloodFlow Analysis [n=3,244] or FMD Studio, Quipu SRL [n=2,118]) which are largely operator independent, and also substantially more reproducible than manual approaches (Woodman et al., 2001). These software packages track the vessel walls and blood velocity trace in B-mode frames via a pixel density and frequency distribution algorithm

(Woodman et al., 2001). An optimal region of interest to be analysed was selected by the sonographer, based on consistent image quality, with a clear distinction between the artery walls and lumen. Despite the initial region of interest selection being operator-determined, the remaining analysis was automated and independent of operator bias (Woodman et al., 2001). Laboratory-specific details of analysis software and ultrasound machines are reported in the appendix.

4.2.3 Statistical Analysis

Statistical analyses were conducted using IBM SPSS version 25 (SPSS Inc., Chicago, IL) unless stated otherwise.

4.2.3.1 Multiple Imputation

Multiple imputation chained equations were used to impute missing values (Janssen et al., 2010) for weight, BMI, systolic-, diastolic- and mean arterial blood pressure, and baseline- and peak diameter (all variables had <20% missing data). Five imputed datasets were generated, which were used to fit the relevant regression models and results reported were obtained from the pooled analyses on all imputed datasets.

4.2.3.2 Definition of Age- and Sex-Related Reference Values

Calculation of age-specific reference intervals were performed in healthy males (n=821) and females (n=582) separately. Initially, to account for differences in analysis software, multiple linear regression was performed, including a dummy variable for FMD Studio as an independent determinant of FMD outcome. The regression coefficient for the dummy variable (β=0.166%) was used as a calibration factor to rescale individual FMD values obtained using FMD Studio. Fractional polynomial (FP) regression (Royston & Wright, 1998) was utilised to calculate age- and sex-specific reference intervals, in STATA

software (Stata Corp., College Station, TX, USA) with the *xrigls* command. Age-specific 2.5^{th}, 10^{th}, 25^{th}, 50^{th}, 75^{th}, 90^{th} and 97.5^{th} percentile curves were calculated as $mean_{FMD}$ + Z_p x standard deviation (SD), where Z_p assumed the values of -1.96, -1.28, -0.67, 0, 0.67, 1.28, and 1.96, respectively. Age- and sex-specific percentile curves were also calculated for baseline brachial artery diameter. Furthermore, Pearson correlation coefficient was used to assess the relationship between baseline diameter and FMD. Fisher r-to-z transformation was used to compare the correlation coefficient between males and females.

4.2.3.3 Relation with CVD Risk Factors

First, the expected $mean_{FMD}$ and SD_{FMD} for individuals with CVD risk factors were calculated based on the equations computed for healthy individuals. Second, age- and sex-specific Z-scores were calculated as $observed_{FMD}$ - $expected_{FMD}$/$SDexpected_{FMD}$. Z-scores represent the number of SDs above or below the healthy population mean (50^{th} percentile) of the same age and sex.

Multiple linear regression determined the relation of CVD risk factors with FMD Z-scores in four subpopulations (un-medicated and medicated males and females). Age was included in the regression model to account for any residual effects on outcomes. Sub-analyses were conducted for smoking and cholesterol, since limited available data were present for these variables. Interaction terms were added between each risk factor and sex to explore whether the effects of the model predictors are moderated by sex differences.

4.3 Results

Participant characteristics are presented for all males and females in Tables 4.1 and 4.2, respectively.

4.3.1 Age- and Sex-Related Reference Values for Brachial Artery FMD

The best fitting FPs' powers (p) for $mean_{FMD}$ and SD_{FMD} were both p=1 for females, which represents a linear relation between FMD and age. For males, the $mean_{FMD}$ p=0 and SD_{FMD} p=-0.5, indicating a curvilinear relation (Figure 4.1). The equations derived for estimated FMD were:

Females:

$mean_{FMD}$ (%) = 9.5947 – 0.0631 x age

SD_{FMD} (%) = 4.5400 – 0.0349 x age

Males:

$mean_{FMD}$ (%) = 7.9279 - 1.5725 x ln(age/10)

SD_{FMD} (%) = 1.4008 + 2.3163 x $(age/10)^{-0.5}$

5.3.2 Age- and Sex-Related Differences in Baseline Brachial Artery Diameter

The best fitting FPs' powers (p) for $mean_{BaselineDiameter}$ and $SD_{BaselineDiameter}$ were p=-2 and p=-1 respectively for females, and p=-0.5 and p=-1 respectively for males, indicating a curvilinear relation in both sexes (Figure 4.1). The equations derived for estimated baseline artery diameter were:

Females:

$Mean_{BaselineDiameter}$ (mm) = 3.3764 – 0.6070 x $(age/10)^{-2}$

$SD_{BaselineDiameter}$ (mm) = 0.6389 – 0.3195 x $(age/10)^{-1}$

Males:

$Mean_{BaselineDiameter}$ (mm) = 5.8692 – 2.9237 x $(age/10)^{-0.5}$

$SD_{BaselineDiameter}$ (mm) = 0.7172 - 0.3177 x $(age/10)^{-1}$

Corresponding reference intervals (percentiles) derived from the above equations for estimated FMD and baseline artery diameter are presented in Table 4.3. Correlation analysis demonstrated weak but statistically significant inverse relationships between observed baseline artery diameter and FMD (female r^2=0.163, male r^2=0.149; both P<0.001), which was not different between sex (Fisher's P=0.697; Figure 4.2).

Table 4.1: Participant characteristics of the total male population, and healthy, un-medicated and medicated male subpopulations.

	Total	Healthy	Un-medicated	Medicated
n	3286	821	1920	545
Age (years)	42±19	26±15	45±17	58±11
Body mass index (kg/m^2)	26.3±5.2	22.2±3.8	27.2±4.6	29.4±5.0
Systolic BP (mmHg)	130±17	118±13	133±17	137±16
Diastolic BP (mmHg)	78±13	70±10	81±12	83±11
Mean arterial pressure (mmHg)	95±14	86±10	98±14	101±13
Total cholesterol [mmol/L (*n*)]	5.1±1.0 (1756)	4.2±0.5 (105)	5.3±1.0 (1252)	4.9±1.1 (399)
LDL cholesterol [mmol/L (*n*)]	3.2±0.9 (1562)	2.3±0.5 (87)	3.4±0.9 (1139)	3.0±1.0 (336)
HDL cholesterol [mmol/L (*n*)]	1.2±0.4 (1613)	1.4±0.2 (89)	1.2±0.4 (1181)	1.2±0.4 (343)
Total-to-HDL cholesterol ratio (n)	4.4±1.3 (1612)	3.0±0.5 (89)	4.5±1.3 (1180)	4.3±1.3 (343)
Triglycerides [mmol/L (*n*)]	1.6±1.1 (1670)	0.9±0.4 (92)	1.6±1.1 (1221)	1.7±1.1 (357)
Plasma glucose [mmol/L (*n*)]	5.5±1.5 (1293)	4.7±0.6 (83)	5.3±1.1 (982)	6.6±2.4 (228)
Baseline artery diameter (mm)	4.37±0.86	3.90±0.83	4.46±0.82	4.75±0.72
FMD (%)	5.56±2.91	6.66±3.24	5.45±2.72	4.30±2.42
Current smoker [*n* (%)]	127 (3.9)	0	102 (5.3)	25 (4.6)
Diabetes [*n* (%)]	288 (8.8)	0	107 (5.6)	181 (33.2)
Dyslipidaemia [*n* (%)]	1474 (44.9)	0	1074 (55.9)	400 (73.4)
BP-lowering medication [*n* (%)]	499 (15.2)	0	0	499 (91.6)
Lipid-lowering medication [*n* (%)]	253 (7.7)	0	0	253 (46.4)
Glucose-lowering medication [*n* (%)]	167 (5.1)	0	0	167 (30.6)

Data are presented as mean±SD. For blood metabolites, *n* represents the number of available data within the respective subpopulation. For categorical data, data are presented as *n* (percentage of subpopulation). BP – blood pressure; HDL – high-density lipoprotein cholesterol; FMD – flow-mediated dilation.

Table 4.2: Participant characteristics of the total female population, and healthy, un-medicated and medicated female subpopulations.

	Total	Healthy	Un-medicated	Medicated
n	2076	582	1247	247
Age (years)	41±18	28±16	44±16	56±12
Body mass index (kg/m^2)	25.7±6.3	21.6±3.5	26.9±6.1	29.5±7.1
Systolic BP (mmHg)	125±19	113±12	129±18	138±18
Diastolic BP (mmHg)	76±12	68±10	78±12	81±14
Mean arterial pressure (mmHg)	92±15	83±10	94±14	98±17
Total cholesterol [mmol/L (*n*)]	5.3±1.0 (1150)	4.3±0.4 (119)	5.1±1.0 (839)	5.3±1.0 (192)
LDL cholesterol [mmol/L (*n*)]	3.3±0.9 (999)	2.3±0.4 (93)	3.4±0.8 (737)	3.2±1.0 (169)
HDL cholesterol [mmol/L (*n*)]	1.5±0.4 (1026)	1.7±0.3 (99)	1.5±0.4 (755)	1.6±0.4 (172)
Total-to-HDL cholesterol ratio (n)	3.6±1.0 (1025)	2.6±0.4 (98)	3.8±1.0 (755)	3.6±1.0 (172)
Triglycerides [mmol/L (*n*)]	1.2±0.9 (1066)	0.8±0.3 (100)	1.2±0.8 (783)	1.5±1.3 (183)
Plasma glucose [mmol/L (*n*)]	5.0±0.9 (866)	4.6±0.5 (107)	5.0±0.7 (656)	5.8±1.6 (103)
Baseline artery diameter (mm)	3.51±0.66	3.25±0.61	3.58±0.64	3.78±0.66
FMD (%)	6.62±3.47	7.78±3.77	6.36±3.22	5.18±3.13
Current smoker [*n* (%)]	97 (4.7)	0	78 (6.3)	19 (7.7)
Diabetes [*n* (%)]	119 (5.7)	0	37 (3.0)	82 (33.2)
Dyslipidaemia [*n* (%)]	883 (42.5)	0	708 (56.8)	175 (70.9)
BP-lowering medication [*n* (%)]	216 (10.4)	0	0	216 (87.4)
Lipid-lowering medication [*n* (%)]	73 (3.5)	0	0	73 (29.6)
Glucose-lowering medication [*n* (%)]	81 (3.9)	0	0	81 (32.8)

Data are presented as mean±SD. For blood metabolites, *n* represents the number of available data within the respective subpopulation. For categorical data, data are presented as *n* (percentage of subpopulation). BP – blood pressure; HDL – high-density lipoprotein cholesterol; FMD – flow-mediated dilation

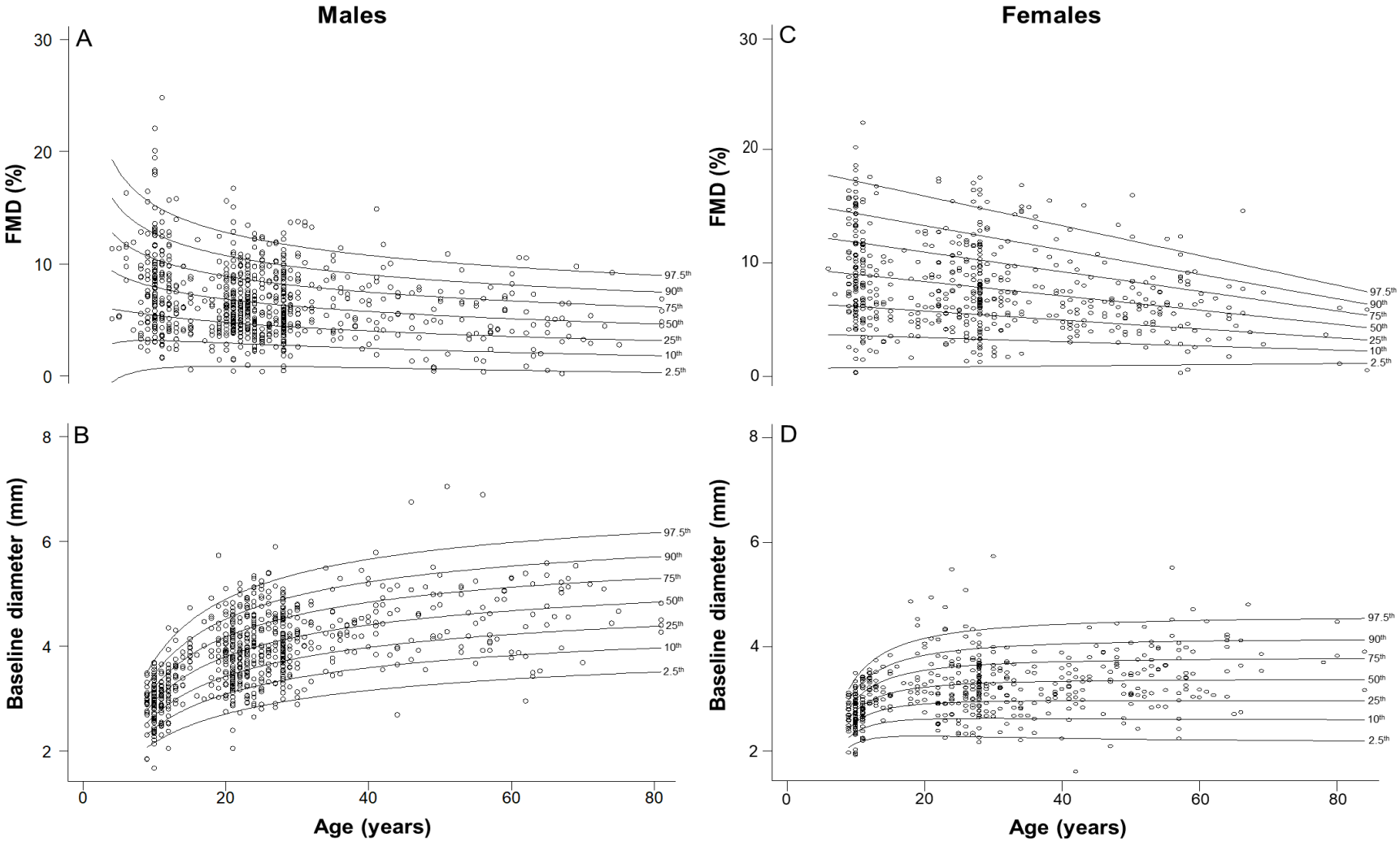

Figure 4.1: Age-specific percentiles of brachial artery flow-mediated dilation (FMD; percentage change from baseline) and baseline diameter (in mm) in males (FMD (**A**) n=821; baseline diameter (**B**) n=790) and females (FMD (**C**) n=582; baseline diameter (**D**) n=571).

Table 4.3: Estimated reference intervals [50th percentile (2.5th-97.5th percentile)] for brachial artery FMD (%) and baseline diameter (mm) in healthy females and males, derived from the predictive equations.

	Age (years)	Females	Males
FMD (%)	5	9.28 (0.72-17.84)	9.02 (-0.15-18.18)
	10	8.96 (0.75-17.18)	7.93 (0.64-15.21)
	15	8.65 (0.78-16.52)	7.29 (0.84-13.74)
	20	8.33 (0.80-15.86)	6.83 (0.88-12.79)
	25	8.02 (0.83-15.21)	6.49 (0.87-12.10)
	30	7.70 (0.86-14.55)	6.20 (0.83-11.57)
	35	7.39 (0.88-13.89)	5.96 (0.79-11.13)
	40	7.07 (0.91-13.23)	5.74 (0.73-10.76)
	45	6.76 (0.93-12.58)	5.56 (0.68-10.45)
	50	6.44 (0.96-11.92)	5.40 (0.62-10.17)
	55	6.12 (0.99-11.26)	5.24 (0.57-9.93)
	60	5.81 (1.01-10.60)	5.11 (0.51-9.71)
	65	5.49 (1.04-9.95)	4.98 (0.46-9.51)
	70	5.18 (1.07-9.29)	4.87 (0.41-9.33)
	75	4.86 (1.09-8.63)	4.76 (0.36-9.16)
	80	4.55 (1.12-7.97)	4.66 (0.31-9.01)
Baseline artery	10	2.77 (2.14-3.40)	2.95 (2.16-3.73)
diameter (mm)	15	3.11 (2.27-3.94)	3.48 (2.49-4.47)
	20	3.22 (2.29-4.16)	3.80 (2.71-4.90)
	25	3.28 (2.28-4.28)	4.02 (2.86-5.18)
	30	3.31 (2.27-4.35)	4.18 (2.98-5.38)
	35	3.33 (2.25-4.40)	4.31 (3.08-5.53)
	40	3.34 (2.24-4.43)	4.41 (3.16-5.66)
	45	3.35 (2.23-4.46)	4.49 (3.22-5.76)
	50	3.35 (2.23-4.48)	4.56 (3.28-5.84)
	55	3.36 (2.22-4.49)	4.62 (3.33-5.92)
	60	3.36 (2.21-4.51)	4.68 (3.37-5.98)
	65	3.36 (2.21-4.52)	4.72 (3.41-6.03)
	70	3.36 (2.20-4.53)	4.76 (3.45-6.08)
	75	3.37 (2.20-4.53)	4.80 (3.48-6.12)
	80	3.37 (2.19-4.54)	4.84 (3.51-6.16)

FMD – flow-mediated dilation.

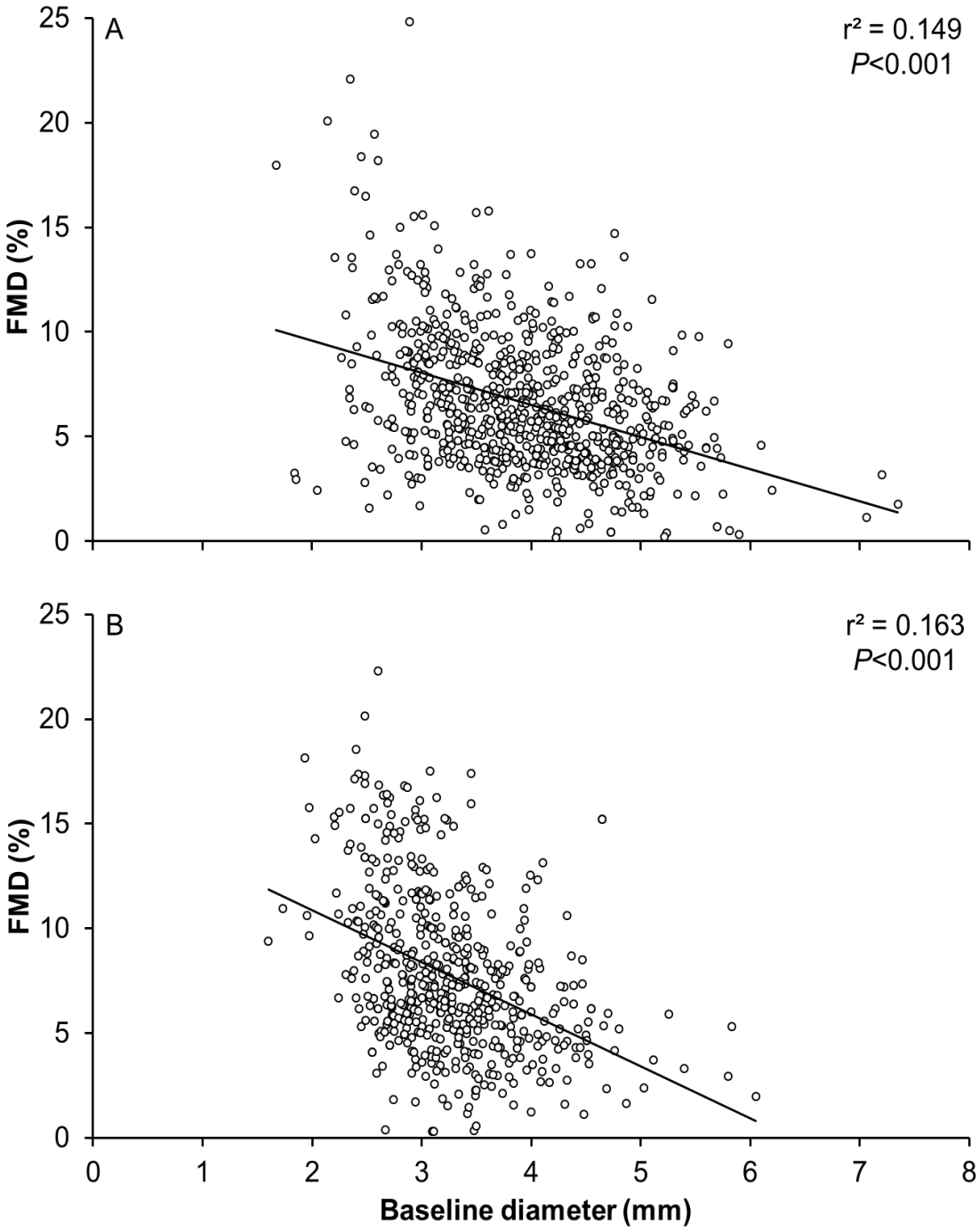

Figure 4.2: Brachial artery FMD and baseline diameter in healthy males (**A**; n=796) and females (**B**; n=579). Pearson correlation coefficient was used to determine the relationship between FMD and baseline diameter in males and females separately.

5.3.3 Relation between CVD Risk Factors and FMD Z-Scores

In the un-medicated subpopulation, lower FMD Z-scores (i.e. lower FMD compared to age-/sex-matched healthy reference values) were found for higher systolic blood pressure in both males and females (P=0.015 and P<0.001, respectively). Higher diastolic blood pressure was significantly associated with higher FMD Z-scores in females (P<0.001). Presence of diabetes was significantly associated with lower FMD Z-scores in males (P<0.001; Table 4.4). In the medicated subpopulation, presence of dyslipidaemia and diabetes were significantly associated with lower FMD Z-scores in females (both P=0.01). In males, smoking and diabetes were significantly associated with lower FMD Z-scores (P=0.022 and P=0.027, respectively), whilst dyslipidaemia was related to higher FMD Z-scores (P=0.029; Table 4.4). These observations are largely reinforced when standardised regression coefficients (per SD increase in- or presence of CVD risk factor) are presented in Figure 4.3.

5.3.4 Sex Differences in the relation between CVD Risk Factors and FMD Z-Scores

Using sex as an interaction term in the regression model revealed that systolic- and diastolic blood pressure were stronger determinants for FMD in un-medicated females than in males (both P=0.019, Figure 4.3). In the medicated subpopulation, no sex differences were found for systolic and diastolic blood pressure (Figure 4.3). Whilst presence of dyslipidaemia was not significantly affected by sex in the un-medicated group, sex altered the effect of dyslipidaemia on FMD Z-score in the medicated group (Figure 4.3). More specifically, FMD was supra-normalised in medicated males, whilst FMD in females was lower in those with dyslipidaemia compared to healthy age- and sex-matched individuals (P<0.001).

Table 4.4: Relation of CVD risk factors with brachial artery FMD Z-scores in un-medicated/medicated males and females.

Sex	Risk Factor	Un-medicated (n=3167)			Medicated (n=792)		
		β	95% CI	P value	β	95% CI	P value
Male	Systolic BP (10mmHg)	-0.049	-0.088; -0.009	0.015	0.017	-0.053; 0.087	0.636
	Diastolic BP (10mmHg)	0.040	-0.013; 0.093	0.136	0.057	-0.049; 0.162	0.293
	Body mass index (kg/m^2)	0.001	-0.009; 0.011	0.788	-0.014	-0.031; 0.004	0.118
	Total-to-HDL cholesterol ratio (unit)	-0.021	-0.068; 0.027	0.391	0.003	-0.084; 0.091	0.942
	Current smoker (yes)	-0.124	-0.332; 0.084	0.241	-0.454	-0.843; -0.066	0.022
	Diabetes (yes)	-0.500	-0.696; -0.303	<0.001	-0.209	-0.393; -0.024	0.027
	Dyslipidaemia (yes)	0.020	-0.077; 0.117	0.682	0.206	0.021; 0.392	0.029
Female	Systolic BP (10mmHg)	-0.117	-0.173; -0.061	<0.001	-0.023	-0.141; 0.096	0.704
	Diastolic BP (10mmHg)	0.150	0.069; 0.231	<0.001	0.124	-0.035; 0.283	0.127
	Body mass index (kg/m^2)	0.004	-0.005; 0.014	0.361	0.010	-0.013; 0.033	0.389
	Total-to-HDL cholesterol ratio (unit)	0.006	-0.073; 0.085	0.879	0.091	-0.096; 0.279	0.339
	Current smoker (yes)	-0.108	-0.344; 0.128	0.368	0.068	-0.474; 0.611	0.805
	Diabetes (yes)	-0.158	-0.492; 0.176	0.354	-0.438	-0.769; -0.107	0.010
	Dyslipidaemia (yes)	-0.075	-0.197; 0.046	0.223	-0.426	-0.752; -0.101	0.010

The regression coefficient β represents the increase in brachial artery FMD (in SD from the healthy population mean of the same age and sex) per unit increase (or presence) in each risk factor. Multivariable regression models including all risk factors and age determined β values. BP – blood pressure; HDL – high density lipoprotein cholesterol.

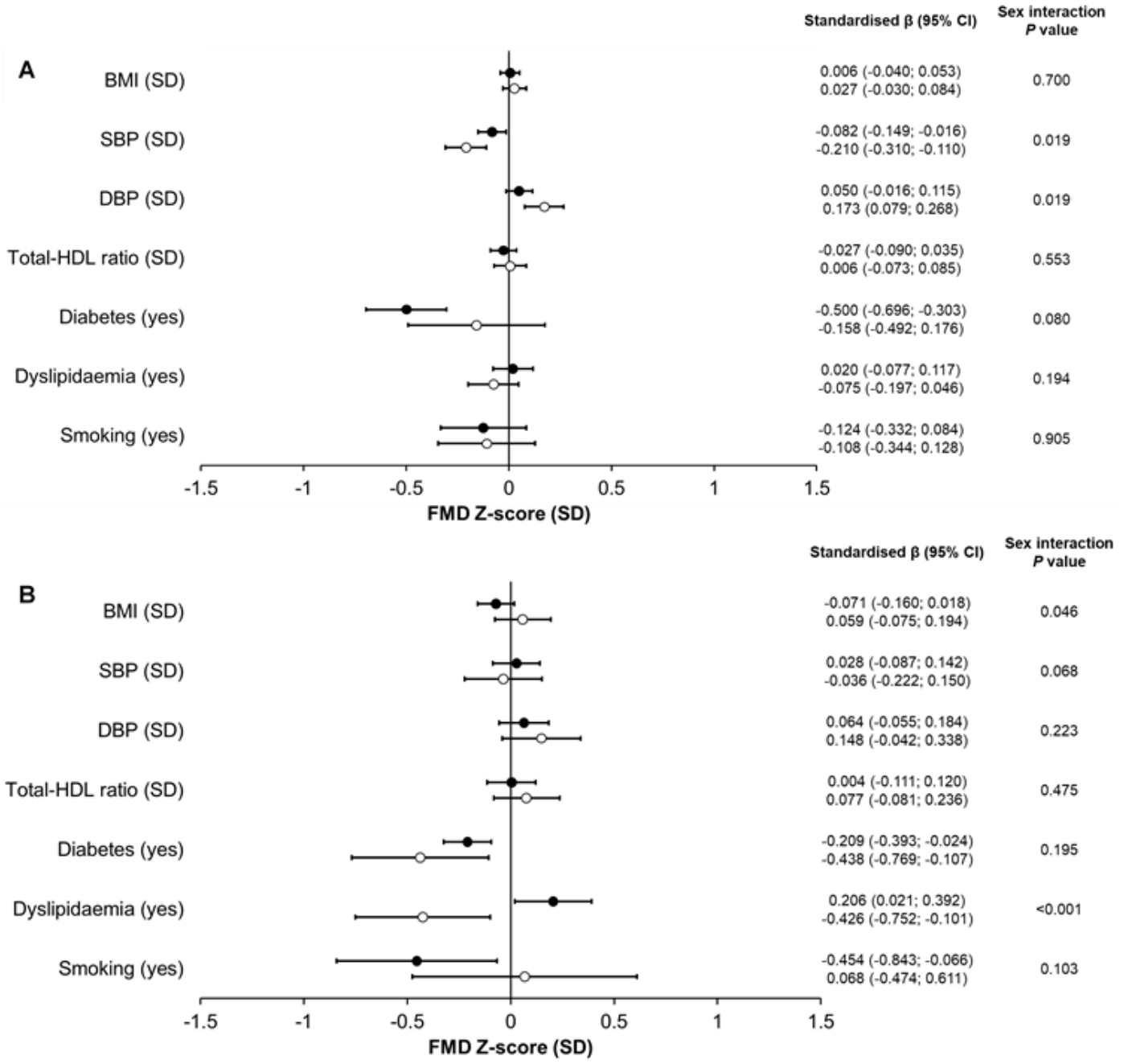

Figure 4.3: Point estimates and 95% confidence intervals represent the increase in brachial artery FMD Z-score (in SD from the healthy population mean) per SD increase (or presence) in risk factor resulting from a multivariable regression model including all risk factors and age for males (●) and females (○). (**A**) un-medicated males (n=1920) and females (n=1247); (**B**) medicated males (n=545) and females (n=247).

4.4 Discussion

Following strict adherence to expert-consensus guidelines (Corretti et al., 2002; Thijssen et al., 2011a), this study provides age- and sex-specific reference intervals for brachial artery FMD, where sex altered the age-related decline in FMD. Healthy males demonstrated a negative curvilinear relation between FMD and age, whilst females revealed a linear relation, where FMD started higher, but declined at a faster rate with age compared to males. Importantly, this work revealed that differences in baseline brachial artery diameter, at least partly, contribute to the age- and sex-related differences in FMD. This suggests that differences in FMD between healthy individuals may not necessarily relate to differences in endothelial function *per se.* Additionally, this work provides insight into how CVD risk factors and (cardiovascular-controlling) medications influence FMD. Some CVD risk factors (e.g. blood pressure, diabetes, dyslipidaemia, BMI) alter age- and sex-related FMD Z-scores, both in un-medicated and medicated individuals. Moreover, sex altered the impact of CVD risk factors and medication. Specifically, a larger impact of blood pressure on FMD was evident in un-medicated females compared to males, whilst dyslipidaemia was associated with a lower FMD in medicated females, but not in males. Taken together, these reference intervals for brachial artery FMD importantly contribute to improved interpretation of FMD outcomes, but also extend the current knowledge and understanding of factors that influence FMD.

In the past years, reference values have been estimated for other (pre)clinical tests of vascular structure (e.g. stiffness (Bossuyt et al., 2015; Engelen et al., 2015) and IMT (Engelen et al., 2013) in large arteries, and media/lumen ratio in small arteries (Bruno et al., 2018)), which contributed to widespread and valid use of the technique. Importantly, in these examples, efforts were made to standardise assessment prior to estimating reference intervals. Similarly, this study included data from laboratories that strictly adhere to guidelines for performance and analysis of brachial artery FMD (Corretti et al.,

2002; Thijssen et al., 2011a). The importance of following these guidelines is supported by this dataset, in that no between-software differences in FMD results were found. Importantly, data were derived from multiple laboratories, different countries, and involved multiple principal investigators and sonographers. This emphasises that adhering to expert-consensus guidelines is essential for the future use of FMD, but also highlights the relevance and robustness of the age- and sex-specific reference intervals presented in this work.

In the healthy population, and in line with most previous work (Adams et al., 1996; Celermajer et al., 1994b; Taddei et al., 1995; Seals et al., 2011), an age-related decline in FMD in both sexes was observed. Nonetheless, the rate of change differed between sexes, with early work reporting a linear decline in both groups that starts around the 4th or 5th decade of life (Celermajer et al., 1994b). Previous studies, however, are limited by the inclusion of a relatively small age range and/or have included individuals with CVD risk factors. Another limitation is largely ignoring the potential role of age- and sex-specific differences in baseline artery diameter, which is relevant since baseline diameter is inversely related to FMD (Thijssen et al., 2008a; Thijssen et al., 2008b; Herrington et al., 2001). Differences in baseline artery diameter may partly explain the lower FMD in males compared to females, and may also influence the age-related changes in FMD. Indeed, the age-related decline in FMD is mirrored by a concomitant increase in baseline diameter. Furthermore, in children there was a steeper rate of change in males compared to females, which may contribute to the characteristic drop in FMD in males (and not in females) during childhood and adolescence.

The higher FMD in females, but also the steeper decline in FMD with age, compared to males may relate to differences in sex hormones, especially since oestrogen has been

linked to cardio-protective properties (Miller & Duckles, 2008), as discussed in *Chapter 2 (Section 2.4)* . Conversely, in contrast with previous research (Moreau et al., 2012a; Celermajer et al., 1994b), the characteristic drop in sex hormones associated with menopause did not translate to a steeper decline in FMD in this study. However, these previous studies are limited by the cross-sectional nature, making it difficult to untangle the impact of menopause *versus* older age. Alternative explanations for the gradual decline in FMD with age may relate to factors including changes in artery structure, shear stress patterns and oxidative stress (see *Chapter 2; Section 2.3*). Future work is required to better understand the nature and physiological mechanisms underlying this change.

When examining the relation between CVD risk factors and FMD Z-scores, blood pressure and diabetes were negatively associated with FMD in un-medicated individuals. This is not surprising, given previous work related to endothelial dysfunction with the presence of high blood pressure (Ghiadoni et al., 2001) and diabetes (Hamilton & Watts, 2013), whilst these risk factors also impacted sex- and age-specific reference values for carotid IMT (Engelen et al., 2013) and arterial stiffness (Engelen et al., 2015; Bossuyt et al., 2015). Moreover, the relation between blood pressure and FMD Z-score disappeared in the medicated subgroup, implying that FMD is not different from healthy controls when using drugs that target these risk factors. These findings are supported by previous work in blood pressure-lowering medication (Taddei et al., 2002), which found these drugs to (in)directly improve endothelial function in patients. No significant impact on FMD in un-medicated individuals was found in other well-established risk factors, including BMI, cholesterol and smoking. A potential explanation for these findings may relate to the small proportion of available data for smoking and cholesterol variables. Nonetheless, the data presented in the current study confirms that elevated blood pressure is an important risk factor associated with endothelial function.

It was also found that sex affects the impact of CVD risk factors and medication on FMD. In un-medicated individuals, systolic- and diastolic blood pressure were stronger determinants of FMD Z-score in females than in males. These findings fit with previous observations, in that untreated hypertensive women showed larger endothelial dysfunction (Routledge et al., 2012) and a stronger relation between hypertension and myocardial infarction incidence compared to men (Anand et al., 2008). Interestingly, sex-specific differences for the effect of blood pressure on FMD disappeared in the medicated group. Additionally, sex differences were reported in the medicated group, with females demonstrating significantly lower FMD Z-scores than males in the presence of dyslipidaemia. In fact, FMD Z-scores for medicated males with dyslipidaemia were supra-normalised (i.e. greater than the healthy population mean of the same age), highlighting the success of drugs targeting dyslipidaemia in males. Whilst the underlying mechanisms for these sex differences remain unclear, these observations are extremely important in contemporary medicine where increased awareness is required that sex differently affects the process of atherosclerosis and CVD development, as well as the impact of pharmacological treatments.

4.5 Conclusion

In conclusion, this study estimated age- and sex-specific percentiles for brachial artery FMD in a healthy population and explored the relation of CVD risk factors on FMD Z-scores. Notably, the FMD data included in the present study were obtained with strict adherence to protocol guidelines (Corretti et al., 2002; Thijssen et al., 2011a). Despite the large number of studies (and contributing authors) included in the analyses, between-study variability was low, emphasising the importance of strict guideline adherence. More importantly, this also highlights the feasibility and use of FMD for (pre)clinical work, when guidelines are strictly adhered to. Accordingly, age- and sex-specific reference values enable better interpretation of FMD outcomes. Moreover, our work also highlights that

sex leads to distinct age-related changes in FMD, but also affects the impact of some CVD risk factors in un-medicated and medicated individuals. Whilst age, sex and CVD risk factors differently affect endothelial function, other physiological factors have been shown to play a role in the FMD outcome. In particular, as discussed in *Chapter 2 (Section 2.5.2)*, the eliciting shear stress stimulus during reactive hyperaemia (i.e. SRAUC) determines the magnitude of FMD (Pyke & Tschakovsky, 2007). Nonetheless, it remains unclear whether this important stimulus is affected by age- and sex-specific differences, and will therefore be the topic of research in the next chapter.

Chapter 5

Age and Sex Differences in the Relationship Between FMD and SRAUC

Published journal article:

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5.1 Introduction

CVD remains the leading cause of morbidity and mortality in women, although the incidence of CVD in women is lower compared to age-matched men (Townsend et al., 2015c). However, a rapid increase in CVD-related mortality in women coincides with the onset of menopause (Townsend et al., 2015c). These sex-related differences in CVD may, at least partly, relate to differences in endothelial function (Green et al., 2016), since pre-menopausal women exhibit enhanced endothelial function compared to men (Celermajer et al., 1994b; Juonala et al., 2008; Yao et al., 2014; Green et al., 2016).

As outlined in *Chapter 2 (Section 2.4)*, these sex-specific differences in FMD largely relates to oestrogen, whereby FMD has shown to fluctuate during a menstrual cycle (Hashimoto et al., 1995; Williams et al., 2001; Brandão et al., 2014) and drop following menopause (Moreau et al., 2012a; Green et al., 2016). An alternative explanation for sex differences in endothelial function relates to observations in animal studies, which suggest that oestrogen improves the vascular responsiveness to changes in shear stress, mediated by oestrogen receptors (Huang et al., 2000). For example, Huang et al. (1998) found that female and ovariectomized rats with oestrogen replacement show significantly greater dilation in response to a given shear stress, compared to male and ovariectomized rats. A stronger relationship between endothelial function and shear stress may therefore contribute to the enhanced endothelial function observed in pre-menopausal women, compared to post-menopausal women and age-matched men. To date, no study has examined this hypothesis in humans.

The purpose of this study was to explore the relationship between FMD and its eliciting shear rate stimulus (i.e. SRAUC) between healthy men and women across the lifespan, and also between pre- *versus* post-menopausal women. It was hypothesised that the

relationship between FMD and SRAUC would be stronger in younger women, compared to men, and that this relationship would be attenuated with older age and post-menopausal status.

5.2 Methods

5.2.1 Participants

Taken from the database detailed in *Chapter 4*, 932 healthy individuals were identified and stratified into young adults (18-40 years; 389 men, 144 women) and older adults (>40 years; 260 men, 139 women) (Table 1). The cut-off level of 40 years was chosen based on the increase in CVD incidence after this age (Roth et al., 2015) and is in line with previous research (Thijssen et al., 2009a). Secondly, based on pre-screening of menopausal status (post-menopause was defined as at least one year without a menstrual cycle/spotting (Moreau et al., 2012a)), sub-analysis was performed between pre-menopausal (*n*=173) and post-menopausal women (*n*=110). All participants were non-smokers, not taking any medication, and free of CVD risk factors and signs or symptoms of cardiovascular or metabolic disease. Pre-menopausal women were not on any hormone-based contraception and post-menopausal women were not on HRT. All participants gave informed consent and all studies were ethically approved by the local ethics committees of Liverpool John Moores School of Sport and Exercise Science Research Ethics Committee, Radboud University Medical Center or The University of Western Australia. All work adhered to the Declaration of Helsinki.

5.2.2 Statistical Analysis

Statistical analyses were performed using SPSS (Version 24, SPSS, Chicago, Illinois). Pearson's correlation coefficient was used to calculate the correlation between FMD and SRAUC across age groups in men and women. This analysis was repeated using the

allometrically scaled FMD to correct for baseline artery diameter (Atkinson & Batterham, 2013a). Fisher r-to-z transformation was used to compare the difference between two correlation coefficients in the independent groups (i.e. sex, age, menopause status). Linear regression analysis was performed to examine the interaction between age-sex group and SRAUC with FMD as the dependent outcome. Other variables (e.g. age, sex, BMI, blood pressure) that have been purported to influence shear rate and/or FMD were also considered in the model. Two-way analysis of variance (ANOVA) was also used to examine the differences between sex and age. Independent t-tests examined the differences between pre- and post-menopausal women. All data were presented as mean±SD, unless stated otherwise. Statistical significance was assumed at $P<0.05$.

5.3 Results

5.3.1 Impact of Age and Sex

Older age was associated with lower FMD, and higher body mass, BMI, systolic, diastolic and mean blood pressure, alongside higher baseline and peak brachial artery diameters (all $P<0.05$). There was a significant main effect for sex, with women demonstrating a lower height, body mass, systolic, diastolic and mean blood pressure, baseline diameter, peak diameter, but a higher FMD response and SRAUC ($P<0.05$; Table 5.1). A significant interaction effect between age and sex was observed for height, body mass, BMI, systolic blood pressure, FMD response and time to peak ($P<0.05$, Table 5.1).

Table 5.1: Subject characteristics of participants divided into young men and women (aged 18-40yrs) and older men and women (>40yrs). Values are mean ± SD. Comparisons between groups were made using a 2-way ANOVA with sex and age as factors.

	Young Adults (18-40yrs)		**Older Adults (>40yrs)**		**ANOVA**		
	Women	*Men*	*Women*	*Men*	*Sex*	*Age*	*Sex*Age*
n	144	389	139	260			
Age (years)	27±6	25±5	56±10	59±10	0.535	<0.001	<0.001
Stature (m)	1.69±0.08	1.80±0.07	1.63±0.07	1.77±0.06	<0.001	<0.001	0.003
Body mass (kg)	69.6±14.0	76.3±10.3	69.7±14.0	82.9±14.1	<0.001	<0.001	<0.001
BMI (kg/m^2)	24.6±5.2	23.6±2.8	25.5±4.5	26.1±4.8	0.130	<0.001	0.030
Systolic blood pressure (mmHg)	113±10	120±11	124±15	127±14	<0.001	<0.001	0.010
Diastolic blood pressure (mmHg)	68±8	72±14	74±9	77±9	<0.001	<0.001	0.907
Mean arterial pressure (mmHg)	86±11	87±11	92±10	94±10	<0.001	<0.001	0.524
Diameter (mm, baseline)	3.3±0.5	4.1±0.6	3.5±0.5	4.4±0.6	<0.001	<0.001	0.218
Diameter (mm, peak)	3.6±0.5	4.3±0.6	3.7±0.5	4.6±0.6	<0.001	<0.001	0.103
FMD%	7.9±3.9	6.4±2.7	5.3±3.3	4.8±2.3	<0.001	<0.001	0.021
SRAUC (s^{-1}, $x10^3$)	23.0±12.0	20.4±10.7	21.6±11.0	19.7±9.0	0.003	0.175	0.662
Time to peak (secs)	51±25	59±30	64±30	58±28	0.575	0.006	0.002

BMI - body mass index; FMD - flow-mediated dilation; SRAUC - shear rate area-under-the-curve.

A significant positive correlation between FMD response and SRAUC was evident in young men (r^2=0.042, *P*<0.001; Figure 5.1A). Young women also demonstrated a significant correlation between FMD and SRAUC (young women r^2=0.112, *P*<0.001), which did not significantly differ compared to young men (Fisher: *P*=0.15). The correlation between FMD and SRAUC was non-significant in older men (r^2=0.011, *P*=0.098), whilst older women presented a very weak, but significant correlation (r^2=0.029, *P*=0.047, Figure 5.1B). Using allometrically scaled FMD, the presence of a correlation in young women (r^2=0.108, *P*<0.001), and a lower correlation in older women were confirmed (r^2=0.029, *P*=0.045), although this difference did not reach statistical significance (Fisher: *P*=0.15). Young and older men did not demonstrate a significant correlation between the allometrically scaled FMD and SRAUC (r^2<0.001 and *P*=0.662*,* r^2<0.001 and *P*=0.779, respectively).

The impact of age, sex and SRAUC on FMD was further investigated using interaction terms in linear regression. This approach revealed evidence of a differential relationship between sex and age status and SRAUC on subsequent FMD outcomes. More specifically, young women demonstrated a significantly stronger relationship between SRAUC and FMD compared to young men (β=-5.8^{-4}, *P*=0.017) and older women (β=-5.9^{-4}, *P*=0.049). Age did not significantly alter the relation between SRAUC and FMD in men (β=-2.5^{-4}, *P*=0.30).

Other variables that might contribute to FMD response were also explored in the linear regression model. In addition to age-sex-SRAUC interactions, FMD is influenced by systolic blood pressure (β=-0.035, *P*=0.001), but not diastolic blood pressure (β=0.006, *P*=0.60) or BMI (β=0.033, *P*=0.26). Given the systolic blood pressure outcome, the bivariate correlations were repeated in a subset of n=505 who all fell within strict cut-off values for normal blood pressure (systolic <130 mmHg, diastolic <80 mmHg), BMI (<25

kg/m^2) and, when available, glucose (<5.6 mmol/L) and cholesterol levels (<4.9 mmol/L). Young men show evidence of a correlation between FMD response and SRAUC (r^2=0.020, *P*=0.024), but this response was significantly stronger in young women (r^2=0.124, *P*<0.001, Fisher: *P*=0.05). Older men and women did not show a correlation between FMD and SRAUC (r^2=0.006 and 0.002, respectively, both *P*>0.05).

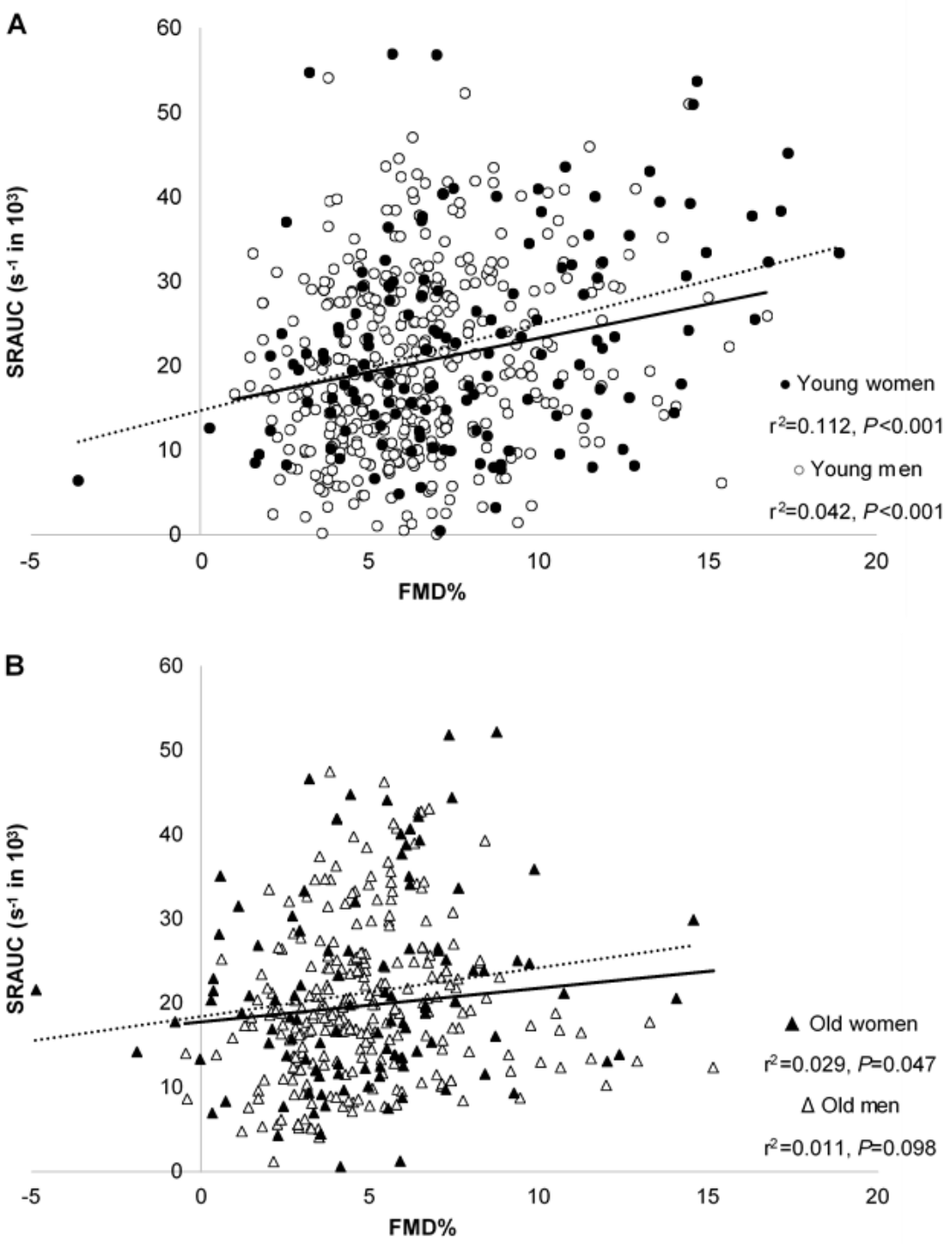

Figure 5.1: Brachial artery FMD (%) and the eliciting SRAUC stimulus (in s^{-1}) in healthy younger (**A**, total n=533) and older (**B**, total n=399) adults. Data were presented and analysed separately for younger men (open circles, n=389) and women (solid circles, n=144), but also for older men (open triangles, n=260) and women (solid triangles, n=139). Pearson's correlation coefficient was used to examine the relation between the FMD and SRAUC in younger and older women (dotted line) and men (solid line).

5.3.2 Impact of Menopausal Status

Compared to pre-menopausal women, post-menopausal women demonstrated a higher BMI and blood pressure, but lower height and FMD (all $P<0.05$, Table 5.2). Pre-menopausal women demonstrated a significant correlation between FMD and SRAUC ($r^2=0.097$, $P<0.001$), whilst this correlation was not significant post-menopause ($r^2=0.025$, $P=0.100$, Figure 5.2, Fisher: $P=0.19$). Using the allometrically scaled FMD, these findings were confirmed as the correlation with SRAUC in pre-menopausal women ($r^2=0.095$, $P<0.001$), disappeared post-menopause ($r^2=0.025$, $P=0.099$, Fisher: $P=0.20$). Re-analysis of the correlation coefficients within the subgroup of healthy participants (n=505) confirmed the presence of a correlation between FMD and SRAUC in pre-menopausal women ($r^2=0.09$, $P=0.001$), which was absent in post-menopausal women ($r^2=0.006$, $P=0.73$, Fisher: $P=0.30$).

Table 5.2: Subject characteristics of women divided based on hormonal status. Values are mean ± SD. P-value refers to an independent t-test.

	Pre-menopause	**Post-menopause**	***P* value**
n	173	110	
Age (years)	30±8	59±9	<0.001
Height (m)	1.68±0.08	1.62±0.07	<0.001
Body mass (kg)	69.6±13.7	70.0±15.1	0.938
BMI (kg/m^2)	24.7±5.1	26.4±5.0	0.007
Systolic blood pressure (mmHg)	113±10	126±14	<0.001
Diastolic blood pressure (mmHg)	69±9	74±9	<0.001
Mean arterial pressure (mmHg)	82±8	90±10	<0.001
Baseline diameter (mm)	3.3±0.5	3.6±0.5	<0.001
Peak diameter (mm)	3.6±0.5	3.8±0.6	0.018
FMD%	7.8±3.9	4.9±3.1	<0.001
SRAUC (s^{-1}, $x10^3$)	23.0±11.6	21.3±11.4	0.213
Time to peak (secs)	51±24	68±32	<0.001

BMI - body mass index; FMD - flow-mediated dilation; SRAUC - shear rate area-under-the-curve.

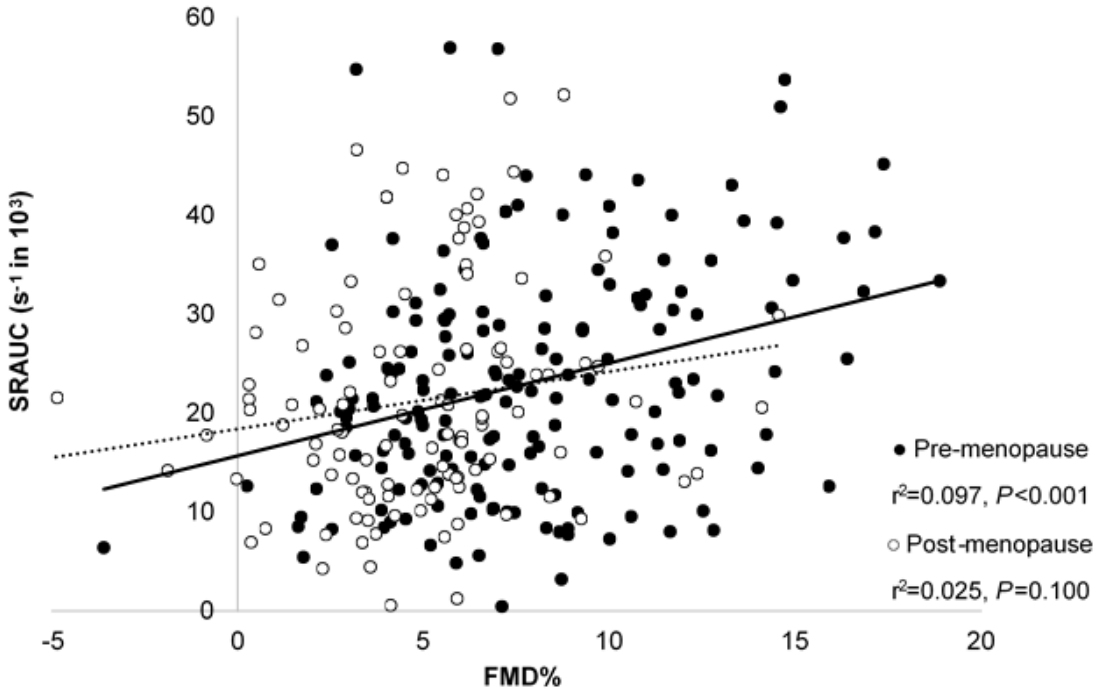

Figure 5.2: Brachial artery FMD (%) and the eliciting SRAUC stimulus (in s^{-1}) in healthy pre-menopausal women (solid circles, n=173) and post-menopausal women (open circles, n=110). Pearson's correlation coefficient was used to examine the relation between the FMD and SRAUC in pre- (solid line) and post-menopausal women (dotted line).

5.4 Discussion

The initial analyses were suggestive of sex differences in conduit artery FMD across the lifespan. However, given the impact of systolic blood pressure on FMD, analysis was repeated on a subset of participants following the American Heart Association guidelines for blood pressure (Whelton et al., 2018). This revealed a significantly stronger relationship between FMD and SRAUC in young women compared to young men, and this was attenuated with advancing age. The sex-related difference and the impact of menopausal status on the relationship between FMD and its eliciting shear stress stimulus suggests that oestrogen may play a role in mediating the higher FMD in pre-menopausal women and, consequently, the reduced risk of CVD in comparison to young men (Townsend et al., 2015c).

This study in a large population of 932 healthy adults confirms previous work on the association between FMD and shear rate, in that a statistically significant correlation is present between endothelial function and the magnitude of the shear stress stimulus. This correlation remained present after correcting the FMD for individual differences in baseline diameter and when performed in a subset of healthy individuals (n=505). Given that SRAUC is the eliciting stimulus of the FMD response (Pyke & Tschakovsky, 2007), one would expect to observe a moderate-strong correlation between the two variables. However, the data presented in the current study shows a somewhat weaker correlation, in general, compared to previous work, especially in men (Thijssen et al., 2009a). This finding could be attributed to a number of participant characteristics, which may lead to a weaker or even absent relation between FMD and SRAUC (e.g. age, CVD risk factors) (Mitchell et al., 2004; Thijssen et al., 2009a). Indeed, the sub-analysis performed within individuals with no risk factors revealed a slightly higher r-value. In addition, other factors that impact upon the FMD response must be acknowledged, such as the response of the vascular smooth muscle cells to dilator signals (endothelium-independent dilation was not assessed in these studies) and the structural properties of the artery (i.e., wall thickness, stiffness and diameter (Koller & Kaley, 1991; Lehoux et al., 2006; Thijssen et al., 2008a)). Also, numerous studies have shown that baseline diameter is a stronger predictor of the FMD response than SRAUC (Thijssen et al., 2009a; Mitchell et al., 2004; Silber et al., 2005; Pyke et al., 2004; Pyke & Tschakovsky, 2007) and the scaling of FMD responses to baseline diameter attempted to account for this.

In line with some previous observations, sex-related differences in the relationship between FMD and the eliciting SRAUC stimulus were observed. More specifically, young healthy women demonstrated a stronger correlation between FMD and SRAUC, compared with their male peers, especially in the healthy subgroup. To examine the potential role of oestrogen, a sub-analysis based on menopausal status was performed

and found that the relationship between FMD and SRAUC was absent in post-menopausal women. The potential cardio-protective properties of oestrogen have been described in *Chapter 2 (Section 2.4)*. These adaptations likely contribute to changes in vascular health, especially since some studies have shown that the cyclical oestrogen levels across the menstrual cycle are mirrored by fluctuations in arterial stiffness (Adkisson et al., 2010; Robb et al., 2009) and endothelial function (Hashimoto et al., 1995; Williams et al., 2001; Brandão et al., 2014; Adkisson et al., 2010). Some of this work used intra-brachial infusions to examine forearm blood flow responses, an endothelial assessment independent of shear rate, and confirmed that endothelial function *per se* fluctuates across the menstrual cycle (Williams et al., 2001). Studies that utilised FMD found that fluctuations in this variable across the menstrual cycle were independent of changes in the shear rate stimulus (Adkisson et al., 2010; Hashimoto et al., 1995; Williams et al., 2001; Brandão et al., 2014). This suggests that these larger FMD responses are explained, at least partly, by enhanced sensitivity of the endothelium to shear rate.

Distinction between levels of oestrogen receptors may contribute to the relationship between FMD and the shear rate stimulus in pre-menopausal women. As detailed in *Chapter 2 (Section 2.4.1.1)*, the abundance of oestrogen receptors is determined by circulating oestrogen (Gavin et al., 2009; Stirone et al., 2003; Pinna et al., 2008; Ihionkhan et al., 2002), which is consequently linked to increased NO bioavailability (Rubanyi et al., 1997; Pinna et al., 2008). Given the important role of NO in CVD protection and vasodilation, it could be suggested that oestrogen receptors mediate the relationship between FMD and shear stress, resulting in greater dilator responses to a given shear stress stimulus. More research is required to explore the mechanisms underlying the FMD-SRAUC relationships observed in the present study.

When exploring the effects of age, the FMD-SRAUC relationship was attenuated with advancing age in both men and women, which confirms previous findings (Thijssen et al., 2009a). Notably, a weak, but significant correlation in was observed in older women. However, this observation may be attributable to the inclusion of 29 (21%) pre-menopausal women in the older (over 40 years) group. These findings therefore provide further evidence that older age impairs the FMD-SRAUC relationship. Various components of vascular ageing (outlined in *Chapter 2, Section 2.3*), including alterations in blood vessel structure, shear patterns and attenuated NO bioavailability may potentially contribute to the age-related attenuation in the FMD-SRAUC relationship. Since these processes are also present in women, one may question the relative importance of age *versus* oestrogen in the loss of the relationship between FMD and SRAUC in post-menopausal women. Given the more gradual impact of age on these factors compared with the relatively rapid alterations in oestrogen, one may hypothesise that the loss of oestrogen may represent a stronger factor than age in explaining the loss of the relationship between FMD and SRAUC. Future studies are required to untangle the effects of age and sex on this relationship.

A potential lifestyle factor underlying the age- and sex-related differences in the FMD-SRAUC relationship relates to fitness and/or physical activity levels. It is well established that physical activity and subsequent fitness is associated with enhanced endothelial function (Hagg et al., 2005; Siasos et al., 2013; Davison et al., 2010) amongst a myriad of other health markers, mediated by the activity-induced exposure to increases in cyclical shear stress (Thosar et al., 2012). Since studies highlight a trend for declining physical activity levels with advancing age (Milanovic et al., 2013; Townsend et al., 2015b), age-related differences in physical activity may represent a confounding variable in the relationship between FMD and SRAUC. Future studies are warranted to better understand this potential link.

5.4.1 Clinical Relevance

The clinical relevance of these findings relate to the importance of changes in shear stress as an important hemodynamic stimulus for acute (Tinken et al., 2009; Greyling et al., 2015a) and chronic (Tinken et al., 2010; Naylor et al., 2011a) adaptation in vascular function and structure (Green et al., 2017). High levels of shear stress have also been linked to the upregulation of anti-atherogenic proteins and down-regulation of pro-atherogenic substances (Green et al., 2017; Newcomer et al., 2011; Fisslthaler et al., 2000) to provide further protection against the development/progression of atherosclerosis. Accordingly, enhanced sensitivity of the endothelium to increases in shear stress (e.g. induced by physical activity) in younger women may contribute to relatively lower risk for CVD events in this cohort. In addition, such changes may also contribute to impaired ability for remodelling of arteries in response to prolonged periods of changes in shear stress in older women. Importantly, shear stress-mediated changes in endothelial function, for example by exercise training, lead to clinically important improvements in vascular health, with a 1% increase in brachial FMD being associated with 8-13% reduction in CVD risk (Ras et al., 2013; Inaba et al., 2010; Matsuzawa et al., 2015).

5.4.2 Strengths and Limitations

Firstly, data on oestrogen levels were not available, which makes it difficult to directly link these observations to menstrual status and/or oestrogen. Furthermore, it must be acknowledged that the timing/duration of menopause may also play a role in mediating the FMD-SRAUC relationship. However, vigorous eligibility screening for the respective study established menopause status. Furthermore, markers of endothelial activation/damage were not available, which may have helped to better understand the age-related changes in endothelial function and/or the role of shear stress. Another limitation is that data were collected in different laboratories, which may contribute to

some variation. Nonetheless, all labs strictly followed expert-consensus guidelines (Thijssen et al., 2011a) and utilised identical data collection and validated software analysis procedures which result in high reproducibility of FMD (Greyling et al., 2016a).

5.5 Conclusion

In conclusion, a stronger relationship between endothelial function and the eliciting shear rate stimulus was found in women, compared to men, with this sex difference being attenuated with advancing age in the healthy subgroup. These findings suggest that endogenous oestrogen may play a role in mediating the relationship between SRAUC and FMD. Therefore, the stronger relationship between endothelial function and shear stress (compared to men) may contribute to the cardio-protection of young women and subsequent lower prevalence of CVD.

Despite the somewhat weak correlation observed in this study, shear stress remains an important physiological stimulus for vasodilation during the assessment of FMD. In addition, shear stress has been shown to mediate vascular structural and functional adaptations, specifically in response to exercise (Green et al., 2017). Previous efforts have been made to examine the impact of different shear rate patterns (i.e. different levels of antegrade and retrograde shear) with various exercise modalities. Nonetheless these interventions importantly caused changes in mean shear stress, making it difficult to assess the isolated effects of antegrade versus retrograde shear. Therefore, the next chapter will explore the impact of increasing antegrade and retrograde shear through fluctuations without altering mean shear stress levels.

Chapter 6

The Effect of Fluctuations in Shear Rate on Brachial Artery FMD

Published journal article:

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6.1 Introduction

Increased mean shear stress represents a key stimulus for vascular adaptation (Green et al., 2017; Tinken et al., 2010; Birk et al., 2012) and *in vivo* evidence has shown that elevation in mean shear rate mediates acute (Tinken et al., 2009; Greyling et al., 2015b) and chronic (Naylor et al., 2011a) improvement in FMD. In addition to levels of mean shear stress, the pattern of shear stress is important, since increasing the antegrade shear component was associated with improved FMD, whilst increasing retrograde and oscillatory shear is associated with impaired FMD (Thijssen et al., 2009c; Schreuder et al., 2015).

As outlined in *Chapter 2 (Section 2.6.2)*, Sundby et al. (2016b) showed that exposure to intermittent negative pressure causes fluctuations in patterns of blood flow and shear rate, whilst this method also improved wound healing in patients with lower limb ischaemia and ulcers (Sundby et al., 2016a; Sundby et al., 2018b; Sundby et al., 2018a). These clinical effects suggest that fluctuations in blood flow and shear stress patterns may impact vascular health in humans. Nonetheless, since these studies did not control for potential increases in mean shear levels, it remains unclear whether these observations are linked to repetitive exposure to fluctuations in shear, or increases in mean shear stress levels.

No previous study in animals or humans has directly examined whether fluctuations in blood flow and shear stress patterns, in the presence of unaltered mean blood flow and shear rate, impacts upon endothelial function. Therefore, the aim of this study was to assess the effect of 30-minute exposure to intermittent negative pressure, which mediates fluctuations in blood flow and shear rate patterns through the brachial artery, on FMD in healthy young men. It was hypothesised that fluctuations in blood flow and

shear stress patterns would induce improvement in brachial artery FMD. Since fluctuations in mean shear stress are relevant to many activities of daily living, this study was planned to provide insight into the potential clinical relevance of fluctuations in shear stress as a hemodynamic stimulus for improvement in vascular health *in vivo*.

6.2 Methods

6.2.1 Participants

Fifteen healthy males (age 27.3±5.0 years) were recruited for the study. All participants were non-smokers, not taking medication and/or supplements known to influence the cardiovascular system and free from cardiovascular/metabolic disease risk factors. Based on a pre-screening health questionnaire, participants were excluded if they had poor circulation (including diagnosis of peripheral vascular disease or Reynaud's disease). Each participant provided written informed consent before taking part in the experimental procedure. The research study was ethically approved by the Liverpool John Moores School of Sport and Exercise Science Research Ethics Committee and adhered to the Declaration of Helsinki.

6.2.2 Experimental Design

Participants completed two conditions (control and intervention; control condition data not shown) on separate days. After 15 minutes of supine resting, bilateral brachial artery FMD was assessed, followed by a 10-minute rest period to allow blood flow and diameter to return to baseline levels. Subsequently, following a 1-minute recording of baseline diameter and blood flow velocity, subjects underwent a 30-minute intervention involving intermittent negative pressure (i.e. left arm), whilst the right arm served as a control arm. Within 2-minutes of this intervention, bilateral brachial artery FMD was repeated.

6.2.3 Preparations

Prior to the laboratory visit, all participants were instructed to refrain from strenuous exercise for at least 24 hours, alcohol for 12 hours, avoid all caffeinated products for 8 hours and food products high in polyphenols for 24 hours. Participants reported to the quiet, temperature-controlled laboratory after fasting for at least 6 hours. Stature and body mass were recorded to the nearest 0.1 unit using a stadiometer and digital scales, respectively. BMI was calculated as body mass in kilograms divided by stature in metres squared (kg/m^2).

6.2.4 Brachial Artery FMD

Brachial artery FMD was measured in accordance with contemporary expert-consensus guidelines ((Thijssen et al., 2011a); see *Chapter 3*). Following 15 minutes of supine rest, left and right brachial artery FMD were assessed simultaneously via high-resolution duplex ultrasound (Terason u-smart 3300, Teratech, Burlington, MA) with a 10-12 MHz linear array probe. All measurements were taken by the same experienced operators within participants. Bilateral FMD was repeated following the 30-minute intervention period.

6.2.5 Brachial Artery Diameter and Shear Rate

High-resolution ultrasound was used to examine brachial artery diameter and shear rate as described above. Following the pre-intervention FMD, the participant's skin was marked to ensure a consistent ultrasound probe position and therefore artery segment during the visit. Furthermore, the ultrasound machine settings remained constant (i.e. depth and Doppler cursor position) in order to assume the same probe angle whilst imaging. Bilateral artery diameter and shear rate were recorded for 1-minute baseline, and repeated at 5-minute intervals during the 30-minute intervention period.

6.2.6 Intervention

During the laboratory visit, participants rested in the supine position with both arms extended away from their body to approximately 80°, with their palms facing upwards for optimal ultrasound imaging of the brachial artery. During the 10-minute rest period following the pre-intervention FMD, the left arm was placed inside a rigid plastic cylinder (8.5x40cm) connected to a pressure control box (FlowOx^{TM}, Otivio AS, Oslo, Norway; Figure 6.1). The cylinder was sealed around the forearm with a thermoplastic elastomer (TPS-SEBS). The arm was exposed to repeated bouts of negative pressure (-40 mmHg; 10 seconds negative pressure, 7 seconds atmospheric pressure) for 30 minutes (~105 full cycles of negative pressure).

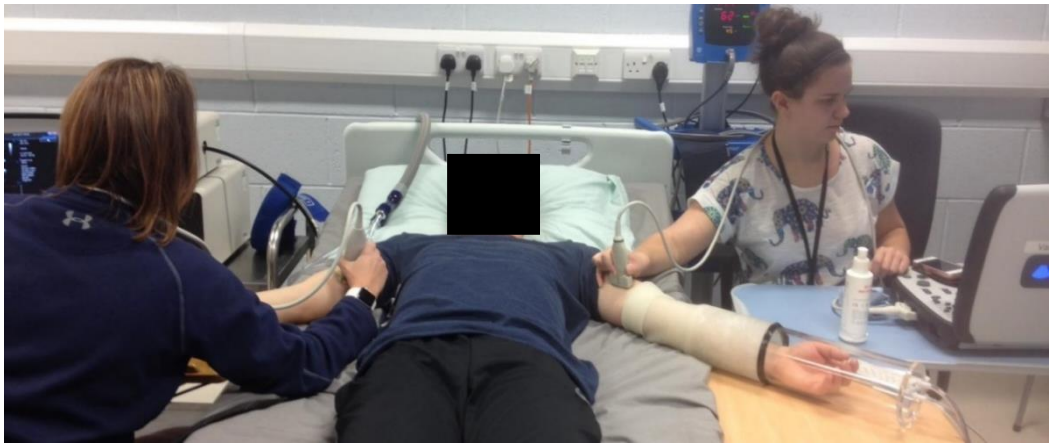

Figure 6.1: Photo of the experimental set-up.

6.2.6.1 Blood Pressure

Blood pressure and heart rate were recorded continuously during the protocol from the right (control) arm index/middle finger using a Portapres (Finapres Medical Systems BV, Amsterdam, The Netherlands). This data were displayed, recorded and exported using PowerLab software (ADInstruments, Australia). The difference in blood pressure and heart rate was calculated from a 1-minute recording before the intervention period started, and the last minute of the intervention.

6.2.7 Data Analysis

All FMD data analysis was performed blinded by the same observer using BloodFlow Analysis software, and in line with protocol guidelines (see *Chapter 3*). Brachial artery diameter and shear rate (during the intervention) were analysed using the same software. The region of interest location (selected by the operator) remained consistent for each 1-minute recording *within* participants. Using markers placed by the operator, the software calculated the average artery diameter and shear rate across the minute recordings. The fluctuations in shear stress were analysed as an average during the application of negative pressure (10secs; On), atmospheric pressure (7secs; Off), and the full cycle, then repeated for the 3 full cycles captured during each 1-minute recording. These processes were repeated for each time point during the intervention (i.e. every 5 minutes). Mean (± standard error) shear rate data at baseline and during 3 cycles of intermittent negative pressure are presented in Figure 6.2.

6.2.8 Statistical Analysis

Statistical analysis was conducted using IBM SPSS version 25 (SPSS Inc., Chicago, IL). Allometric scaling was performed on FMD data to control for differences in baseline diameter (Atkinson & Batterham, 2013a; Atkinson et al., 2013). A linear mixed model with covariate control for SRAUC and scaled baseline diameter determined the main effect for time (pre-post) and arm. A general linear model assessed the changes in blood pressure and heart rate across the intervention period. Paired T-tests determined the difference in antegrade and retrograde shear during intermittent negative pressure compared to baseline in both arms. Statistical significance was recognised when a *P* value <0.05 was observed. Data are presented as mean ± standard error unless stated otherwise.

6.3 Results

Subject characteristics are presented in Table 1.

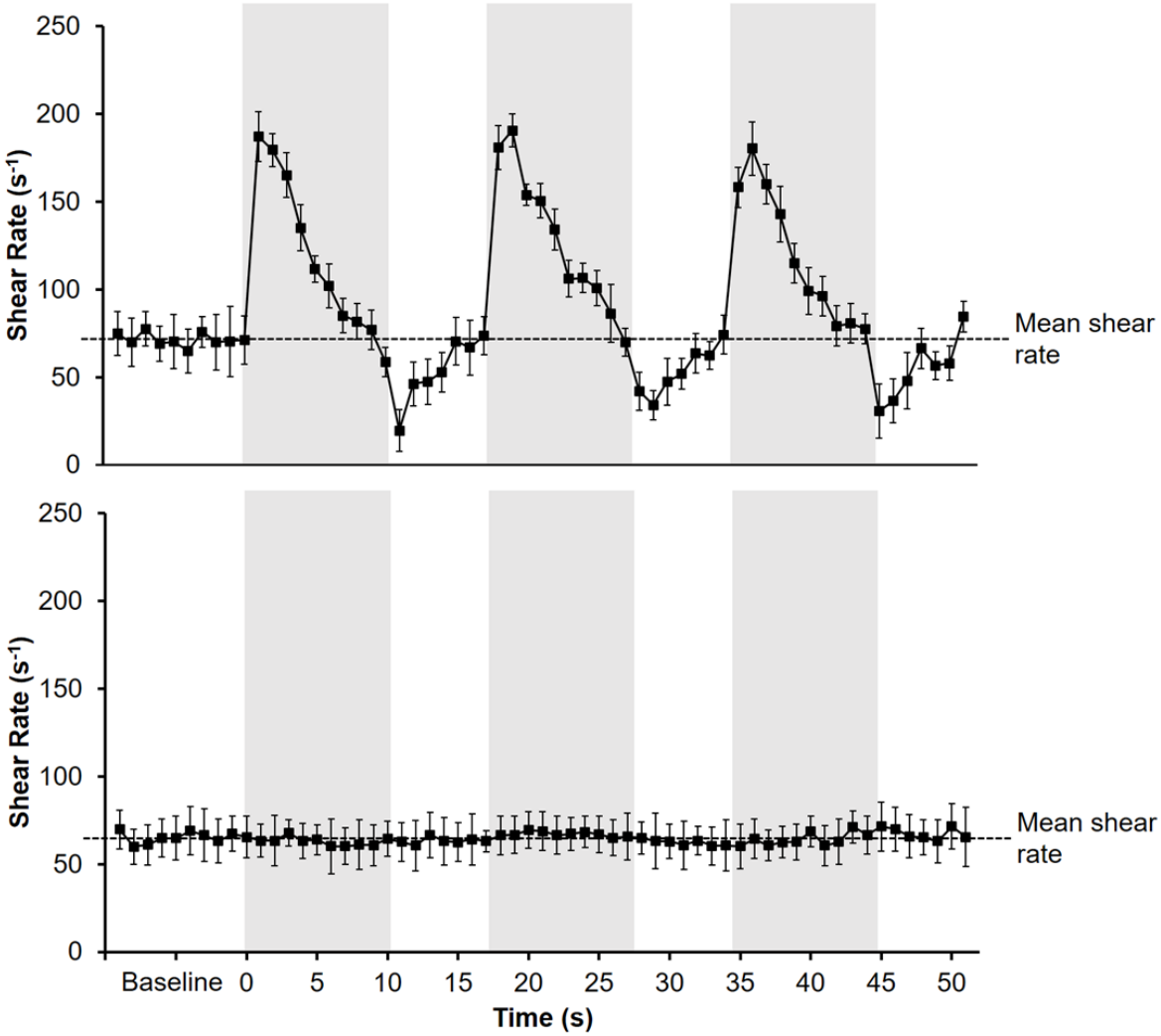

Figure 6.2: Shear rate data in the intervention arm (**A**) and control arm (**B**), calculated as 1-s averages at rest, followed by 3 cycles of intermittent negative pressure (grey bars: negative pressure). Values are mean ± standard error. Mean shear rate is presented as the dashed line.

Table 6.1: Subject characteristics of the participants (n=15).

Parameter	Mean±SD
Age (years)	27.3±5.0
Stature (m)	1.75±0.06
Body mass (kg)	75.1±7.5
Body mass index (kg/m^2)	24.4±2.0
Systolic blood pressure (mmHg)	115±3
Diastolic blood pressure (mmHg)	62±7
Mean arterial pressure (mmHg)	80±5
Resting heart rate (bpm)	52±8

6.3.1 Brachial Artery Blood Flow and Shear Rate

There were no significant changes across the 30-minute intervention in heart rate (mean 52bpm ± SD 7 bpm *versus* 54±8 bpm, *P*=0.47) or in systolic (129±9 mmHg *versus* 135±12 mmHg, *P*=0.16), diastolic (55±8 mmHg *versus* 59±9 mmHg, P=0.36) or mean arterial blood pressure (80±8 mmHg *versus* 84±9 mmHg, *P*=0.23). Negative pressure was associated with a significant increase in mean shear rate, whilst pressure release was followed by a significant decrease in mean shear rate, to levels below baseline ("pressure on": Δ+34.2s^{-1}, "pressure off": Δ-26.5s^{-1}; both *P*<0.001; Figure 6.3A). Consequently, mean shear rate across the intervention period was not different from baseline ("pressure on/off cycle": Δ+3.8s^{-1}; *P*=0.458). In the control arm, negative pressure did not change mean shear from baseline levels ("pressure on": Δ+1.6 *P*=0.805, "pressure off": Δ+3.5s^{-1} *P*=0.613). Therefore, mean shear rate remained unchanged throughout the intervention period compared to baseline ("pressure on/off cycle": Δ+2.5s^{-1} *P*=0.702; Figure 6.3B).

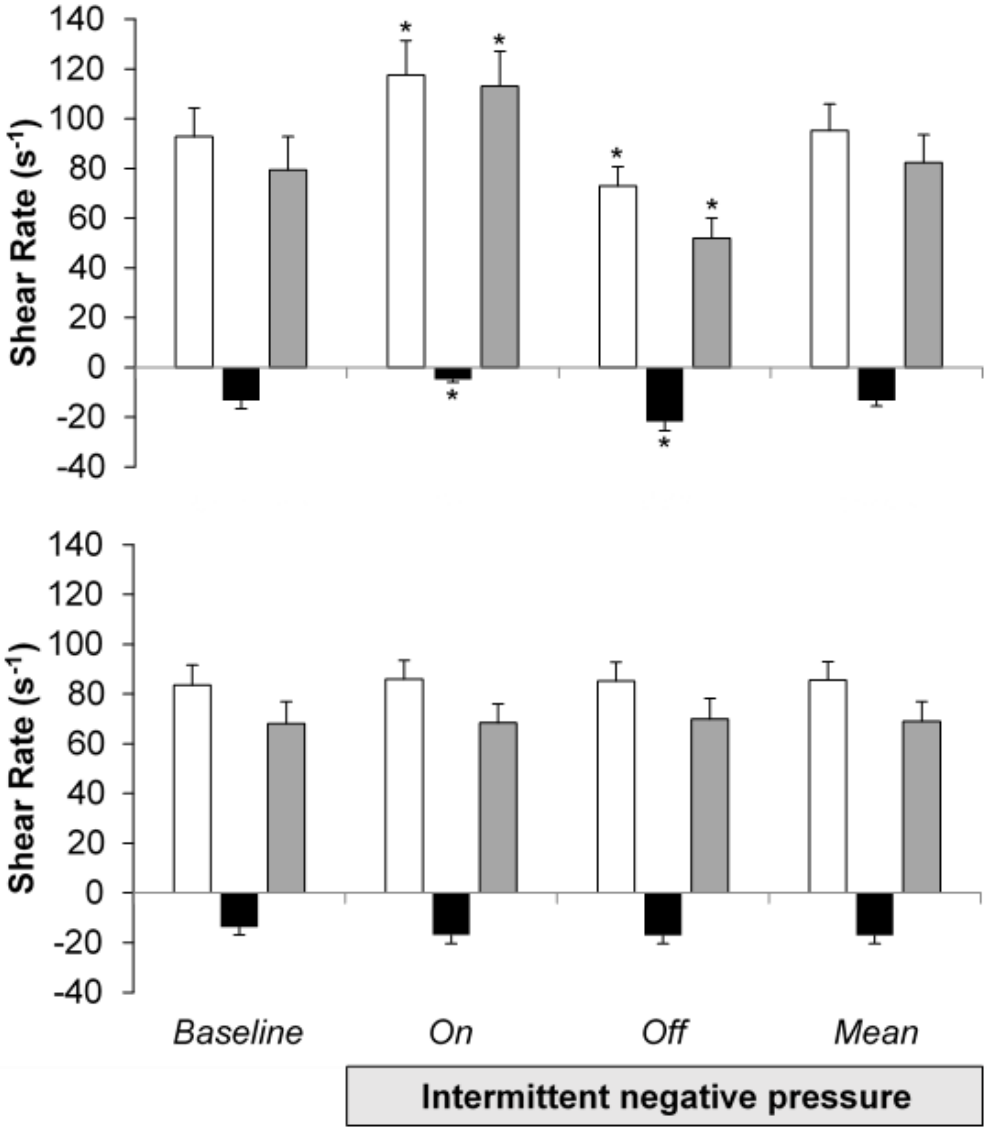

Figure 6.3: Average levels of antegrade (white bars), retrograde (black bars) and mean (grey bars) shear rate at baseline and during the intermittent negative pressure intervention in the intervention arm (**A**) and control arm (**B**). Data during the intermittent negative pressure were presented during negative pressure ('on'), during pressure release ('off') and as the average across the entire 30-minute intervention ('mean'). Error bars represent standard error. Paired T-tests determined differences in shear rate compared to baseline. *Significantly different from baseline at P<0.05.

When examining shear patterns, negative pressure increased antegrade shear rate (*P*<0.001) and decreased retrograde shear rate (*P*=0.006; Figure 6.3A). Upon pressure release, compared to baseline levels, a decrease in antegrade shear rate and increase in retrograde shear rate was found (*P*=0.003 and *P*<0.001, respectively). As a result, mean antegrade and retrograde shear rate across the 30-minute intervention period was

not different from baseline (*P*=0.504 and 0.777, respectively). Antegrade and retrograde shear rate remained unaltered from baseline in the control arm during “pressure on” (antegrade: Δ+2.5s^{-1}, *P*=0.730; retrograde: Δ-1.9s^{-1}, *P*=0.190) and “pressure off” (antegrade: Δ+1.9s^{-1}, *P*=0.779; retrograde: Δ-2.0s^{-1}, *P*=0.164; Figure 6.3B). Therefore, mean antegrade and retrograde shear rate was not different from baseline across the intervention (antegrade: Δ+2.2s^{-1}, *P*=0.750; retrograde: Δ-1.9s^{-1}, *P*=0.173).

6.3.2 Brachial Artery FMD

Linear mixed model analysis revealed a significant main effect for time (*P*=0.029; F-ratio=5.146), whilst no effect was observed for arm (*P*=0.619; F-ratio=0.251) or interaction effect (*P*=0.096; F-ratio=2.906). Post-hoc exploratory analysis (LSD) revealed a significant increase in FMD in the intervention arm (Δ+2.0%, *P*=0.008), whilst no change was observed in the control arm (Δ+0.5%, *P*=0.664). Individual FMD responses are presented in Figure 6.4 and all associated parameters (mean and 95% confidence intervals) are presented in Table 6.2.

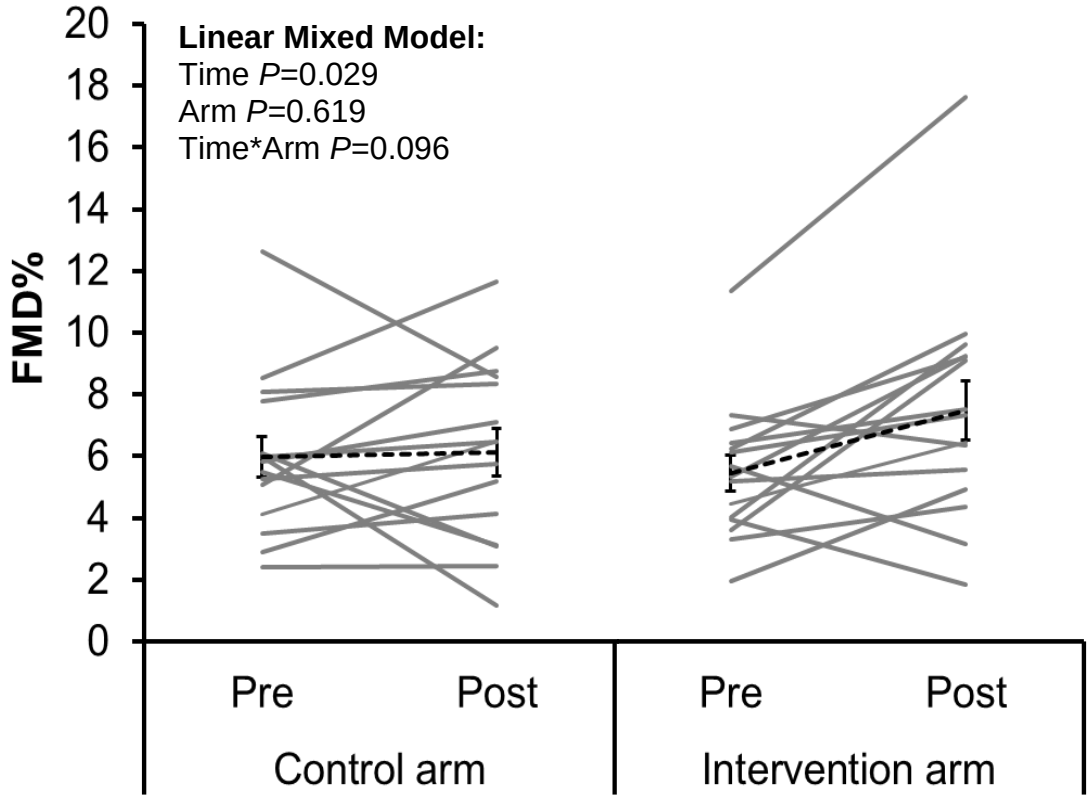

Figure 6.4: Individual brachial artery FMD responses to 30-minutes intermittent negative pressure in the intervention and control arms of healthy young individuals (n=15). Black dotted line represents mean change in FMD. Error bars represent standard error. A linear mixed model determined the main effect for time and arm.

Table 6.2: Brachial artery FMD for the intervention and control arms before and after 30-minute exposure to unilateral intermittent negative pressure. *P*-values refer to a linear mixed model to examine the main effect of 'time' (pre- *versus* post-intervention), 'arm' (intervention-arm *versus* contra-lateral control arm) and the interaction-effect between 'time'*'arm'. Data are presented as mean (95% confidence intervals).

	Intervention arm		**Control arm**				
	Pre	*Post*	*Pre*	*Post*	**'time'**	**'arm'**	**'time*arm'**
Baseline diameter (mm)	4.04 (3.82-4.26)	4.02 (3.79-4.24)	3.82 (3.60-4.05)	3.79 (3.57-4.01)	0.671	0.002	0.957
Peak diameter (mm)	4.26 (4.03-4.48)	4.31 (4.09-4.54)	4.07 (3.84-4.30)	4.05 (3.82-4.27)	0.797	0.001	0.603
FMD (%)	5.5 (3.9-7.0)	7.5 (5.9-9.0)	6.4 (4.9-8.0)	6.9 (5.4-8.5)	0.029	0.619	0.096
SRAUC ($s^{-1}x10^3$)	19.3 (15.0-23.5)	17.9 (13.6-22.1)	17.1 (12.8-21.3)	17.5 (13.2-21.7)	0.762	0.428	0.572
Time to peak (secs)	48 (40-56)	43 (35-51)	43 (35-51)	47 (39-55)	0.950	0.919	0.217

FMD – flow-mediated dilation; SRAUC – shear rate area-under-the-curve.

6.4 Discussion

This study showed that application of intermittent negative pressure to the forearm increases antegrade blood flow and shear rate, whilst pressure release mediates increased retrograde blood flow and shear rate measured at the brachial artery, relative to baseline and the contralateral control arm. Despite these marked fluctuations in blood flow and shear rate patterns throughout the 30-minute intervention, mean blood flow and shear rate was not different from baseline. Therefore average resting levels of flow and shear rate were successfully preserved, despite inducing fluctuations of these variables. Although exploratory in nature, brachial artery FMD improved as a result of these fluctuations in blood flow and shear rate, an effect that was not apparent in the contralateral control limb. Taken together, these findings suggest that fluctuations in shear rate, independent of mean blood flow and shear rate, may impact acute vascular function in healthy young individuals. Whilst further research is required, this contributes to improving understanding of shear stress as an important hemodynamic stimulus in the adaptation of vascular health in humans *in vivo.*

These findings regarding the impact of cyclical negative pressure are in line with a previous study in the lower limbs (Sundby et al., 2016b). Importantly, the current study adds the novel knowledge that these fluctuations were associated with improvements in endothelial function, as measured with the brachial artery FMD. Blood pressure and heart rate remained unaltered during the intervention period, effectively excluding the possibility that systemic factors contributed to these observations. To further support this notion, no changes in brachial artery blood flow or shear rate were found in the contralateral arm. This strongly suggests that the mechanisms contributing to the increase in FMD in the intervention arm relate to local effects (i.e. fluctuations in shear rate) rather than systemic/circulating factors.

These novel results may be somewhat surprising, in that the fluctuations in shear rate were not accompanied by changes in mean shear rate, but still caused an increase in FMD. In previous work, supported by studies in animals (Pohl et al., 1986; Woodman et al., 2005), it has been consistently reported that changes in mean shear rate are essential to change FMD (Thijssen et al., 2009c; Tinken et al., 2009). More specifically, selective increases in antegrade shear rate (and therefore mean shear rate) were related to improved FMD (Tinken et al., 2009; Greyling et al., 2015b), whilst an isolated increase in retrograde shear rate (i.e. lower mean shear rate) was associated with a dose-dependent decrease in brachial and femoral artery FMD (Thijssen et al., 2009c; Schreuder et al., 2015). One potential explanation for the increase in FMD is the relative larger importance of increases in antegrade shear rate compared to changes in retrograde shear rate. To support this idea, moderate-intensity cycling exercise acutely increases retrograde shear rate (Green et al., 2002; Thijssen et al., 2009b), followed by normalisation after ~15 minutes with a concomitant increase in antegrade shear rate (Simmons et al., 2011). Nonetheless, acute or chronic performance of cycling exercise (i.e. 30-/40-min bouts) leads to improvement in brachial artery FMD (Green et al., 2017; Birk et al., 2012). This evolving hypothesis that changes in antegrade shear rate may be relatively more important than changes in retrograde shear rate has been discussed further in *Section 7.3.2.2* and warrants further investigation

Another explanation for these findings relates to the importance of fluctuations in shear rate patterns, rather than mean shear rate. In the microcirculation, previous work used mathematical simulation to support the concept that fluctuations of capillary blood flow, rather than steady-state conditions, improve oxygenation of tissue (Tsai & Intaglietta, 1993a). Follow-up work in humans examining skin perfusion and oxygenation demonstrated that periodic fluctuations in vasomotion may be beneficial for local oxygenation (Thorn et al., 2011). In conduit arteries, some studies have found that

enhanced external counterpulsation increased shear rate fluctuations and FMD in the brachial artery (Braith et al., 2010; Gurovich & Braith, 2013). However, these changes were also accompanied by an overall increase in mean shear rate, making it impossible to isolate the impact of fluctuations *per se* (i.e. in the absence of changes in mean shear). Finally, indirect support for a potential clinically-relevant, beneficial effect on vascular health for these fluctuations is provided by the observation of improved wound healing upon repeated exposure to intermittent negative pressure (Sundby et al., 2016a; Sundby et al., 2018b). These observations may contribute to improved microcirculatory blood flow and therefore the delivery of oxygen and nutrients to promote wound healing (Sundby et al., 2018a; Sundby et al., 2016a). Although speculative, findings of the current study suggest that these benefits of intermittent negative pressure stimulus on wound healing (Sundby et al., 2016a; Sundby et al., 2018b) may be related to enhanced endothelial function.

A final possible explanation for these findings relates to the impact of intermittent negative pressure on changes in the pressure gradient across the artery wall (Smyth, 1969) and, therefore, transmural pressure (Pfitzner, 1976). Although changes in transmural pressure may affect vascular health (Green et al., 2017; Atkinson et al., 2015b), it seems unlikely this can explain the present findings. First, negative pressure likely increases transmural pressure (due to the drop in external pressure), which is typically associated with impaired vascular health (Atkinson et al., 2015b). Secondly, vascular function was examined in the brachial artery, i.e. not directly exposed to the changes in transmural pressure, no significant systemic effects on blood pressure of unilateral forearm suction were observed.

6.4.1 Clinical Relevance

The clinical relevance of these findings is that fluctuations in blood flow or shear rate *per se* represent a hemodynamic stimulus capable of improving vascular health. Previous studies manipulating shear rate have increased mean shear rate to improve FMD. In contrast to these stimuli, mean shear rate was not changed, but still found improved FMD, most likely due to the fluctuations in shear and blood flow patterns. Furthermore, these fluctuations in blood flow and shear rate may be more ecologically valid compared to sustained increases in shear rate. More specifically, fluctuations in blood flow and shear rate are more related to activities of daily living, such as those associated with low-intensity physical activity and changes in posture. Therefore, repetitive exposure to these stimuli may be efficient in improving vascular health. Indeed, recent work has demonstrated that regular exposure to mild physical activity stimuli, such as walking breaks (Carter et al., 2018; Thosar et al., 2015) or fidgeting (Morishima et al., 2016), prevents decline in cerebro- and cardiovascular health associated with prolonged sitting. Although speculative, activity-induced fluctuations in blood flow may be the underlying mediator contributing to the preserved vascular health.

6.4.2 Strengths and Limitations

The present study possesses several strengths, including strict adherence to contemporary expert-consensus guidelines for FMD (Thijssen et al., 2011a) and blinded data analysis using custom-designed edge-detection software to eliminate operator bias. There are some limitations to the study. Firstly, healthy recreationally active males were recruited, which makes it difficult to extrapolate these findings to other populations (e.g. females (Hashimoto et al., 1995; Williams et al., 2001; Brandão et al., 2014) or clinical groups). However, larger improvements in FMD may be observed in those with *a priori* endothelial dysfunction (Maiorana et al., 2003). A second limitation is that additional measurements such as blood analysis for markers of endothelial cell activity were not

performed. *In vitro* studies in cultured endothelial cells and isolated arteries, reviewed elsewhere (Green et al., 2017), demonstrate the release of pro- and anti-atherogenic substances in response to exposure to oscillatory (or low) and laminar (or high) shear stress, respectively. Insight into the impact of fluctuations in shear stress (with preserved mean shear) would have contributed to further understanding the underlying mechanisms of the present findings. A final limitation relates to the relatively small sample size of this study. More specifically, 15 participants were selected based on previous studies in which the impact of shear stress manipulation was assessed and demonstrated significant changes in conduit artery diameter and FMD (Thijssen et al., 2009c; Tinken et al., 2009; Tinken et al., 2010; Birk et al., 2012; Carter et al., 2013; Atkinson et al., 2015a; Atkinson et al., 2015b; Naylor et al., 2011a). Nonetheless, post-hoc statistical power analysis using G*Power software (Faul et al., 2007) revealed a power of 0.77 to detect within-subject changes in FMD, but a power of 0.27 to find a significant interaction effect. Therefore, since more participants would be required for greater statistical power, these results should be interpreted with caution, and further work is required to better understand the potency of fluctuations in shear rate patterns on vascular function.

6.5 Conclusion

In conclusion, findings of the current study suggest that 30-minutes exposure to fluctuations in shear rate improves endothelial function, despite the absence of concomitant changes in mean shear rate compared to resting baseline levels. This work implies that fluctuations in blood flow or shear rate may represent a hemodynamic stimulus to potentially improve vascular health. Future research to examine the underlying mechanisms and potential long-term effects would be of interest.

Chapter 7

Synthesis of Findings

7.1 Aims and Objectives

The research presented in this thesis aimed to examine age- and sex-specific differences in brachial artery FMD across the lifespan in healthy individuals. Importantly, through collating data obtained with strict adherence to expert-consensus guidelines, work in this thesis was able to, for the first time, estimate reference values for brachial artery FMD. Since shear stress is a key stimulus for vasodilation, this work also focussed on the role of shear stress in vascular function. More specifically, this thesis examined age- and sex-specific differences in the relation between FMD and SRAUC, in addition to the relevance of shear patterns (i.e. fluctuations in shear rate) for vascular function.

7.2 Major Findings

7.2.1 Sex Differences in Brachial Artery FMD Across the Lifespan

The age- and sex-specific brachial artery FMD reference intervals presented in *Chapter 4* revealed sex differences in the age-related decline in FMD in healthy individuals. Firstly, females demonstrated a consistently larger FMD but declined at a faster rate compared to males, and the pattern of change across the lifespan also differed between sexes. More specifically, females demonstrated a linear relation between FMD and age, compared to a curvilinear relation in males. *Chapter 4* also demonstrated sex differences in age-related increases in baseline brachial artery diameter. These findings suggest that the sex-specific age-related decline in FMD is likely to be partially explained by age-related increases in baseline artery diameter. Notably, since the healthy population were selected following strict inclusion criteria for CVD risk factors (including BMI, blood pressure and blood metabolites), the decline in FMD occurs as a result of healthy human ageing. Furthermore, whilst previous work has highlighted the detrimental effect of CVD risk factors on FMD, including hypertension (Ghiadoni et al., 2001) and diabetes (Hamilton & Watts, 2013), it remains unclear how CVD risk factors influence FMD in comparison to healthy individuals of the same age and sex.

7.2.2 The Relation Between CVD Risk Factors and FMD and the Impact of Sex

Chapter 4 demonstrated that diabetes and systolic blood pressure were significant determinants negatively associated with FMD (i.e. lower FMD compared to healthy individuals of the same age and sex) in both un-medicated males and females. In medicated individuals, diabetes and dyslipidaemia were negatively associated with FMD in males and females, whilst the relation between systolic blood pressure and FMD disappeared (i.e. not different from healthy). This implies that the use of medication targeting these risk factors effectively normalises FMD and therefore improves CVD risk.

When examining sex differences in the relation between risk factors and medications with FMD, *Chapter 4* revealed that systolic- and diastolic blood pressure were stronger determinants of FMD in un-medicated females compared to males. However, in medicated individuals, there were sex differences in the relation between BMI and dyslipidaemia with FMD. More specifically, FMD appeared to be supra-normalised in medicated males with dyslipidaemia (i.e. greater than the healthy population mean), whilst a significant negative relation remained in females. With the novel knowledge of age-, sex- and CVD risk factor-specific differences in FMD, other physiological determinants of FMD outcome must be considered. Specifically, potential age- and sex-specific differences in the eliciting shear stress stimulus.

7.2.3 Age and Sex Differences in the Relation Between FMD and SRAUC

Data presented in *Chapter 5* (published in *Journal of the American Heart Association;* doi: 10.1161/JAHA.118.010994) provided evidence for a stronger relation between FMD and the eliciting shear stress stimulus (i.e. SRAUC) in healthy young women compared to men, whilst this relation was attenuated with older age in both sexes. Nonetheless,

female-specific analysis revealed no statistical differences in the relation between FMD and SRAUC in pre- *versus* post-menopausal women. These findings suggest that women may be more sensitive to increases in shear stress as a stimulus for vasodilation compared to men, but this sensitivity declines with age. Despite the weak relation between FMD and SRAUC, shear stress remains an important stimulus during FMD, and may also be relevant to change/improve FMD.

7.2.4 Shear Stress as a Stimulus to Improve FMD

The acute experimental study detailed in *Chapter 6* (published in *Journal of Applied Physiology;* doi: 10.1152/japplphysiol.00009.2019) demonstrated that FMD can be improved through fluctuations in shear rate (induced by intermittent negative pressure with significant increases in both antegrade and retrograde shear rate) in healthy young men. Importantly, this localised improvement in FMD was observed in the absence of changes in mean shear rate compared to resting baseline levels. This novel data highlights the importance of shear stress as a hemodynamic stimulus for vascular health and function.

7.3 General Discussion

7.3.1 Sex Differences in Vascular Health and Function

7.3.1.1 The Effect of Sex Hormones on FMD

Key physiological differences between males and females relate to the presence and impact of sex hormones, including oestrogen, progesterone and testosterone. The actions of endogenous oestrogen provides protection against CVD and promotes vascular health in women (see *Section 2.4.1.1*), emphasised by the exponential increase in CVD incidence in women at menopausal age (Townsend et al., 2015c). Previous work

in women has demonstrated a menopause-related decline in FMD (Celermajer et al., 1994b; Taddei et al., 1996; Gavin et al., 2009; Moreau et al., 2012a; Moreau & Hildreth, 2014). In contrast to these findings, the reference values data (*Chapter 4*) did not explicitly demonstrate a drop in FMD around menopause, however there was a large overlap of age between pre- and post-menopausal women. When women were grouped by menopausal status, there was no difference in the correlation between FMD and age (Fisher P=0.197; Figure 7.1), whilst FMD differed between groups (pre-menopause 7.7±3.5%, post-menopause 5.9±2.8%; *P*<0.001, Table 7.1). Since menopause generally occurs between ages 40-60 years, additional analysis within this age range showed no difference in FMD between pre- and post-menopausal women (6.5±2.8% and 6.1±2.6%, respectively; *P*=0.47, Table 7.1).

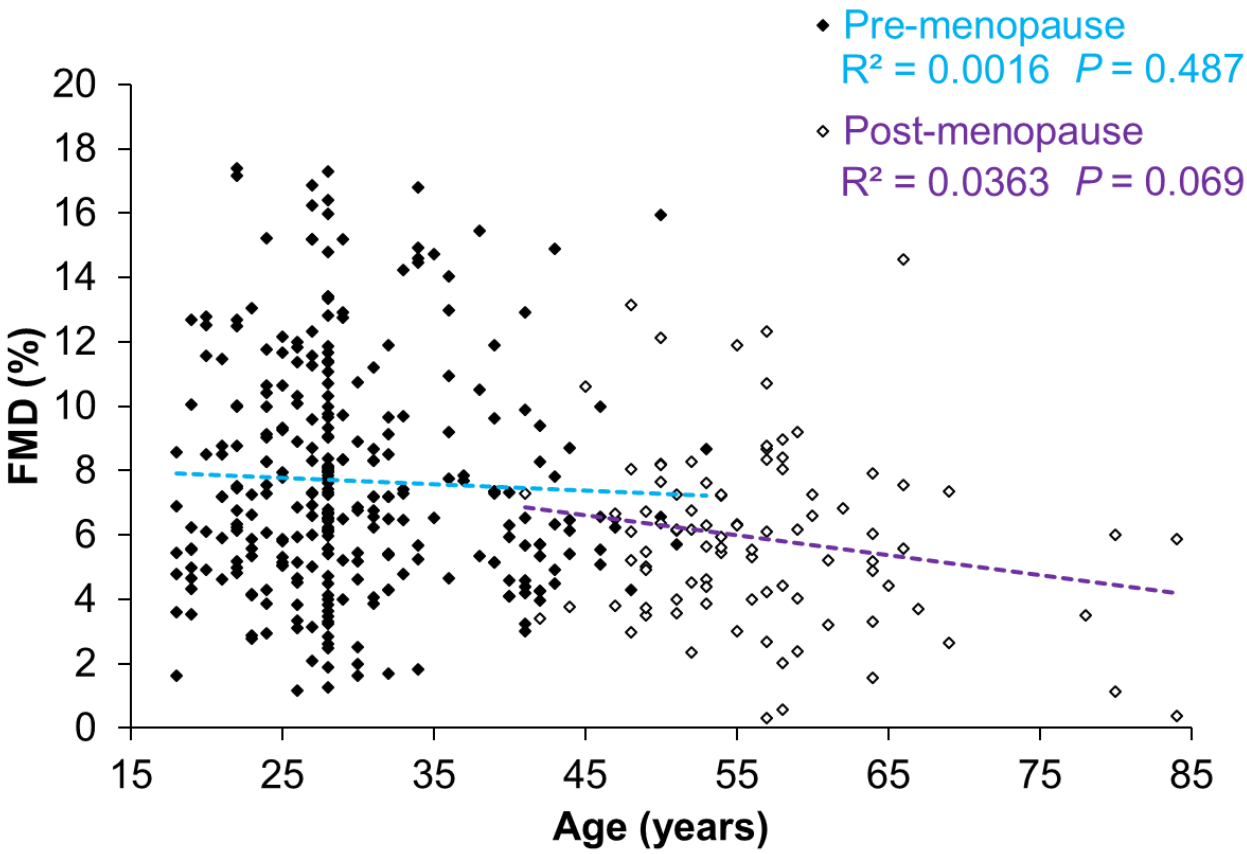

Figure 7.1: Correlation between FMD and age in healthy female adults, organised by hormonal status (pre-menopause [n=303; closed diamonds] and post-menopause [n=92; open diamonds]).

Potential explanations for these discrepancies in comparison with previous literature may relate to the presence of CVD risk factors. More specifically, compared to the female participants included in the reference values dataset, some of the post-menopausal participants assessed by others demonstrate higher BMI (Moreau et al., 2012a; Gavin et al., 2009), waist circumference, and cholesterol (Moreau et al., 2012a). Other CVD risk factors potentially affecting these findings relates to physical activity and fitness. However, these data were not included in the database, and there is inconsistent evidence surrounding the vascular effects of physical activity in old women (Seals et al., 2019; Gliemann & Hellsten, 2019). Taken together, it is plausible to suggest that the menopause-related drop in FMD observed by others (Celermajer et al., 1994b; Taddei et al., 1996; Gavin et al., 2009; Moreau et al., 2012a) may be related to the combined

effect of oestrogen and CVD risk factors associated with the menopause rather than the loss of oestrogen alone.

Another important factor to consider is ageing, since menopause occurs at a different age between individuals. Given that FMD declines with age (*Chapter 4*), the age-related differences may also explain the decline in FMD observed across the menopause transition. Hence, one may question the relative contribution of ageing *versus* menopause in the decline in vascular function in women. In order to control for age-related differences in FMD and (attempt to) isolate the effect of the loss of oestrogen, additional sub-analysis (T-Test) was completed to compare FMD in 11 pairs of age-matched pre- and post-menopausal women, who were also closely matched for BMI and blood pressure to control for CVD risk factors (Table 7.1). A potentially clinically meaningful difference in FMD was observed (pre-menopause 6.97±3.22% *versus* post-menopause 4.95±1.82%; *P*=0.096, Table 7.1). However, given the small sample size, this data must be interpreted with caution. Also, the time spent post-menopause is unknown, which has been shown to negatively affect FMD (Vitale et al., 2008), and the youngest post-menopausal woman was 42 years, immediately presenting greater CVD risk compared to post-menopausal women >45 years (Gong et al., 2016; Muka et al., 2016). Furthermore, a recent study by (Brislane et al., 2019) found no difference in brachial artery FMD between healthy, active late pre-menopausal (~46years) and early post-menopausal (~50years; <5years post-menopause) women. Therefore, the true effect of the menopause on vascular function is likely multi-factorial, but further investigation is warranted to untangle the isolated effects of age *versus* hormonal status on vascular function in women.

Similar to the menopause, puberty occurs at a different age between individuals. Importantly, the large dataset in *Chapter 4* reported brachial artery FMD across the lifespan (4-84 years), whereas previous work has explicitly focussed on children/adolescents (Hopkins et al., 2015) or adults (Juonala et al., 2008). Separate FP regression analyses in adults revealed a negative linear relation between FMD and age in both sexes (Figure 7.2). However, when children were included, this relation became curvilinear in males, but remained linear in females (*Chapter 4, Figure 4.1*). Specifically, FMD in males dropped sharply during childhood and adolescence, followed by a gradual decline across adulthood. The large variation in FMD observed in children may be related to differences in growth and/or maturation, supported by sex-specific differences in artery diameter during childhood (*Chapter 4, Figure 4.1*), where male children exhibited a steeper rate of change in artery diameter compared to females. This may be explained by sex hormones; i.e. testosterone promotes muscle and bodily growth (Rogol, 2002), which in turn may influence vascular structure. As outlined in *Section 2.4.2*, specific underlying mechanisms of puberty and endothelial function remain unclear, and may be related to changes in CVD risk factors such as body composition and blood pressure (Moran et al., 2008). Hopkins et al. (2015) reported significant associations between artery diameter and body size in children, implying that artery size may be mediated by growth/maturation. However, since sex hormone data in addition to Tanner stage was not collected in these studies, it remains unclear how increases in sex hormones *versus* growth, in addition to a reduction in sex hormones (i.e. menopause), directly impacts vascular health and function.

Table 7.1: Subject characteristics and brachial artery FMD data (mean±SD) for pre- and post-menopausal women.

	All women			Age 40-60 years			Age-matched		
	Pre	Post	*P* value	Pre	Post	*P* value	Pre	Post	*P* value
n	303	92		43	71		11	11	
Age (years)	30±7	57±8	<0.001	43±3	53±4	<0.001	46±4	46±4	1.000
BMI (kg/m^2)	21.7±2.1	22.1±1.8	0.123	22.5±1.5	22.1±1.8	0.224	22.1±1.4	22.8±1.5	0.265
Systolic BP (mmHg)	112±10	123±11	<0.001	120±10	123±10	0.169	118±8	119±11	0.851
Diastolic BP (mmHg)	69±9	75±8	<0.001	76±9	76±8	0.868	74±7	74±10	0.818
MAP (mmHg)	83±9	91±8	<0.001	91±9	92±8	0.663	89±8	89±10	0.925
Total cholesterol (mmol/L)	4.3±0.4	4.4±0.4	0.233	4.3±0.4	4.4±0.4	0.625	4.1±0.5	4.3±0.4	0.415
LDL cholesterol (mmol/L)	2.3±0.4	2.4±0.4	0.252	2.2±0.5	2.4±0.4	0.215	1.9±0.6	2.3±0.4	0.280
HDL cholesterol (mmol/L)	1.7±0.3	1.7±0.2	0.832	1.8±0.4	1.7±0.2	0.298	1.7±0.3	1.7±0.3	0.857
Total-HDL ratio (unit)	2.6±0.4	2.7±0.4	0.355	2.5±0.4	2.7±0.4	0.229	2.5±0.5	2.6±0.6	0.761
Triglycerides (mmol/L)	0.8±0.3	0.8±0.3	0.687	0.7±0.3	0.8±0.3	0.670	0.8±0.4	0.7±0.3	0.744
Plasma glucose (mmol/L)	4.6±0.5	4.7±0.6	0.469	4.6±0.4	4.7±0.6	0.693	4.6±0.5	4.5±0.3	0.716
Baseline diameter (mm)	3.35±0.58	3.69±0.65	<0.001	3.44±0.54	3.64±0.65	0.092	3.36±0.53	3.68±0.58	0.205
Peak diameter (mm)	3.60±0.59	3.90±0.67	<0.001	3.66±0.57	3.86±0.67	0.109	3.60±0.57	3.86±0.59	0.317
FMD (%)	7.68±3.50	5.89±2.75	<0.001	6.51±2.83	6.12±2.65	0.470	6.97±3.22	4.95±1.82	0.096

BMI – body mass index; BP – blood pressure; MAP – mean arterial pressure; LDL – low density lipoprotein; HDL – high density lipoprotein; FMD – flow-mediated dilation.

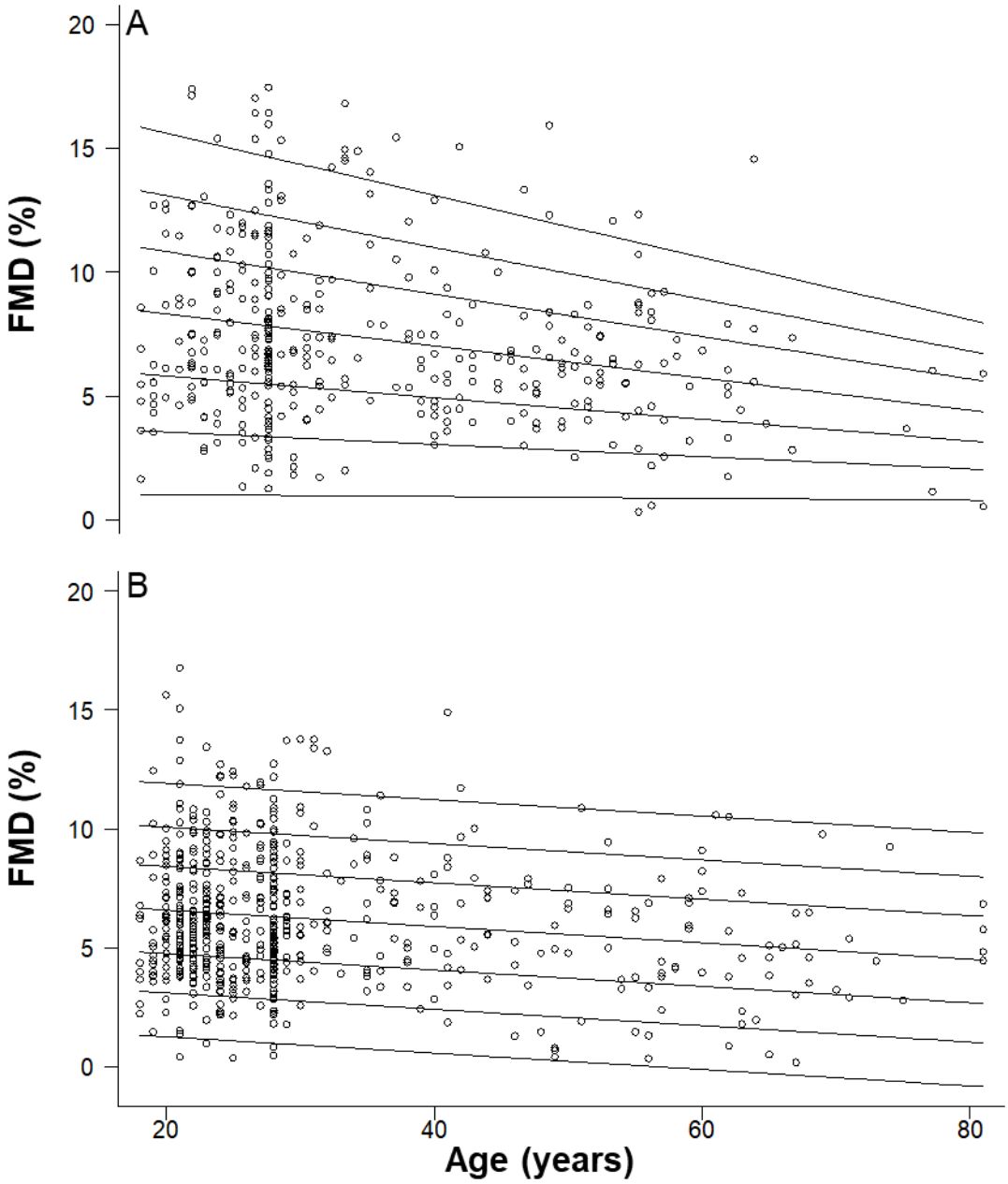

Figure 7.2: Age-specific percentiles of brachial artery FMD in adult females (**A**; n=395) and males (**B**; n=598).

7.3.1.2 Sex Differences in Artery Size

Another important sex difference relates to artery size, which may explain some part of the differences in FMD. *Chapter 4* demonstrated a larger baseline brachial artery diameter in males compared to females, supported by Adams et al. (1996), which also increases across the lifespan, possibly due to structural remodelling in response to age-

related changes in arterial stiffness and vascular tone (Bossuyt et al., 2015; Engelen et al., 2015). A curvilinear relation between artery diameter and age was observed in both sexes, however the rate of change during childhood and adolescence was greater in males compared to females. As discussed above, this may represent different growth/maturation rates between boys and girls. Interestingly, the pattern of change in baseline artery diameter mirrored the curvilinear relation between FMD and age in males, but not in females. Additional correlational analyses revealed a weak relationship with no sex differences between observed FMD and baseline artery diameter (*Chapter 4, Figure 4.2*). However, a very strong relation was observed between estimated FMD and baseline artery diameter (calculated from the equations derived in *Chapter 4*) in males, whilst females demonstrated a weaker relation (Figure 7.3). This data suggests that the age-related increases in baseline artery diameter may play a larger role in the age-related decline in FMD in males compared to females.

The age-related changes in baseline brachial artery diameter may be associated with structural changes within the artery wall, i.e. IMT (Nishiyama et al., 2008), stimulating outward remodelling to preserve lumen diameter. It was proposed by Folkow et al. (1958) that vascular responsiveness was determined by the wall-to-lumen ratio (i.e. the relative artery wall thickness for a given lumen diameter). This concept suggested that smaller arteries (with a greater wall-to-lumen ratio) were “hyper-responsive” to vasoactive stimuli. Accordingly, in support of this hypothesis, a significant correlation was observed between wall-to-lumen ratio and both FMD and GTN responses (Thijssen et al., 2011d). Nonetheless, since wall-to-lumen ratio is generally not measured/reported in FMD literature and was not considered in the reference values dataset, this concept to support the findings presented in *Chapter 4* remains inconclusive.

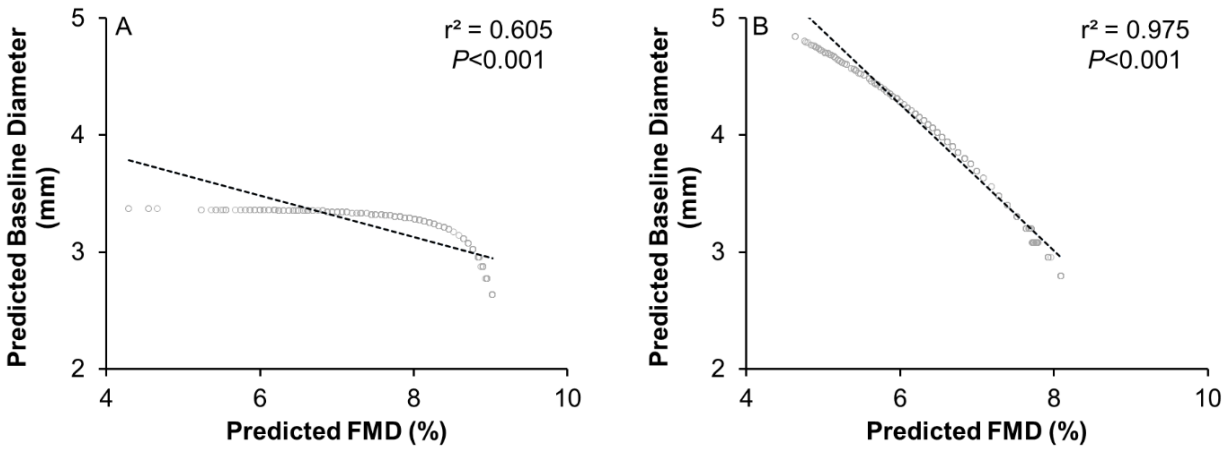

Figure 7.3: Correlation between estimated FMD and baseline artery diameter in females (**A**; n=579) and males (**B**; n=796).

Despite the relevance of baseline artery diameter in FMD, previous work has suggested that peak artery diameter may represent a better marker of conduit artery structure compared to baseline diameter (Naylor et al., 2005b). The authors proposed that it may be more physiologically relevant to assess maximal dilation, since resting (baseline) diameter is influenced by competing vasodilators and constrictors. Interestingly though, when FP analyses were conducted for peak artery diameter (Figure 7.4), the pattern of change was similar to the age-related increase in baseline artery diameter (Figure 4.1). Unsurprisingly, sex-specific differences were evident in peak artery diameter (i.e., larger in males compared to females, and differences in the pattern or change). Although it is likely that the data presented in Figure 7.4 does not represent maximal dilation (compared to GTN and/or ischaemic exercise (Naylor et al., 2005b)), the similarities in the age-related changes in baseline and peak artery diameter highlight the importance of conduit artery structure for determining FMD.

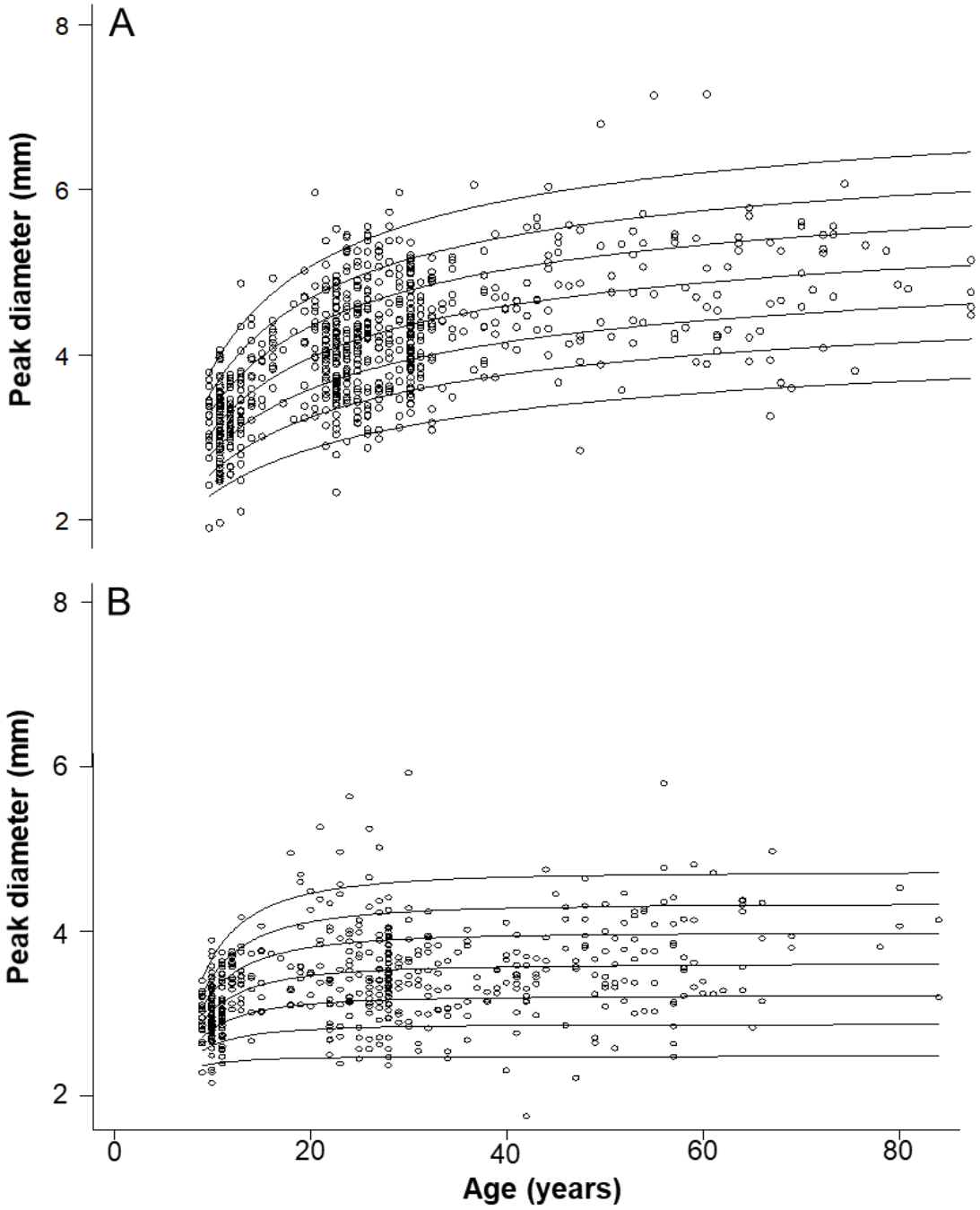

Figure 7.4: Age-specific percentiles of peak artery diameter in healthy males (**A**, n=790) and females (**B**, n=572).

7.3.1.3 Eliciting Shear Stress Stimulus

The total shear stress stimulus (i.e. SRAUC) following cuff release determines the magnitude of vasodilation during the FMD test (Pyke & Tschakovsky, 2007). *Chapter 5* suggested that the sensitivity of the endothelium to shear stress is greater in women compared to men, and declines with age in both sexes. Interestingly, a main effect for sex was observed, where women showed higher SRAUC compared to men. Whilst this may be unsurprising given the smaller artery diameter in women and inclusion of diameter in the calculation of shear rate, no main effect was observed for age (Table 5.1). Moreover, despite significant differences in baseline diameter and FMD, there was no difference in SRAUC between pre- and post-menopausal women (Table 5.2). Therefore, our data shows that pre-menopausal women demonstrated a larger vasodilation response to a given shear stress stimulus, supporting previous work in animals (Huang et al., 1998). Underlying mechanisms have been discussed in *Chapter 2 (Section 2.4.1.1)*, nonetheless these findings support the concept of oestrogen improving the sensitivity of the endothelium to shear stress.

These findings may translate to beneficial vascular adaptations to exercise training. More specifically, functional and structural adaptation induced by repetitive exercise-induced increases in shear stress (Tinken et al., 2010; Green et al., 2017). Nonetheless, whilst the current literature reports favourable vascular effects of regular exercise in ageing men (Seals et al., 2019; Pierce et al., 2011; Tanaka et al., 2000; DeSouza et al., 2000), consensus has not been reached in women (Seals et al., 2019; Witkowski & Serviente, 2018). Some studies have reported no change in FMD in healthy post-menopausal women following an aerobic exercise training intervention (Pierce et al., 2011; Swift et al., 2014; Moreau et al., 2013; Nyberg et al., 2014), whilst others have reported improved FMD only in women with increased CVD risk (Swift et al., 2012). Furthermore, FMD was similar between young and old fit women, which was greater than old sedentary women

(Black et al., 2009), suggesting a protective effect of exercise and/or cardiorespiratory fitness on vascular function with age. Interestingly, previous work in animals (Tarhouni et al., 2016) and humans (Moreau et al., 2013) suggests that the presence of oestrogen mediates the vascular adaptations to exercise in women. It is thought that oestrogen and exercise-induced shear stress share common signalling pathways to facilitate NO production through the activation of eNOS (Traub & Berk, 1998; Kim et al., 2008; Chambliss & Shaul, 2002; Zhang et al., 2009; Chen et al., 1999). *In vitro* studies have shown that oestrogen augments the vasodilation response to a given shear stress stimulus (Huang et al., 1998; Huang et al., 2000), which appears to be mediated by oestrogen receptors (Huang et al., 2000). Since oestrogen concentration determines receptor expression (Gavin et al., 2009), the lack of effect of exercise observed in post-menopausal women may be related to the reduced expression of oestrogen receptors and eNOS activation. Therefore, it is possible that this explains the attenuated relation between FMD and SRAUC in post-menopausal women observed in *Chapter 5*. Nonetheless, evidence surrounding the true vascular effects and underlying mechanisms of exercise in post-menopausal women remains inconclusive and warrants further investigation.

7.3.2 The Role of Shear Stress in Vascular Health and Disease

The vascular tree is comprised of a network of macro- and microvessels, including arteries, arterioles, veins, venules and capillaries. Importantly, these blood vessels are lined with a thin layer of endothelial cells, which are sensitive to hemodynamic forces exerted by flowing blood, i.e., blood pressure and shear stress (Cahill & Redmond, 2016). Given the pivotal roles of the endothelium and shear stress in the development and progression of atherosclerosis (McLenachan et al., 1991; Cecchi et al., 2011), the FMD test is a widely-used research tool to evaluate macrovascular (i.e. conduit artery) endothelial function. The release of the pneumatic cuff during the FMD test induces a

reactive hyperaemic response following distal limb ischaemia to restore blood flow and oxygen delivery. This is quantified as SRAUC as the eliciting stimulus for vasodilation (Pyke & Tschakovsky, 2007), and largely reflects dilation of resistance vessels by ischaemia-induced production of vasodilators including NO (Meredith et al., 1996). Some studies have implied that reactive hyperaemia, measured as forearm blood flow via laser Doppler flowmetry, may represent microvascular function (Higashi et al., 2001), which is attenuated with CVD risk (Binggeli et al., 2003; Rossi et al., 2011; Huang et al., 2007). However, whilst there appears to be a weak relation between forearm blood flow and FMD (Ibrahimi et al., 2018), there is no strong evidence to confirm whether SRAUC measured during the FMD is correlated with microvascular function. Furthermore, microvascular dysfunction often precedes macrovascular dysfunction (Krentz et al., 2007; Sena et al., 2013), and is associated with CVD and diabetes (Jaap et al., 1994; Jaap et al., 1997; Sprague & Ellsworth, 2010). Since *Chapter 4* demonstrated that the presence of diabetes was the strongest determinant negatively affecting FMD, future research should explore the potential to combine macro- and microvascular function to determine endothelial function across different vascular beds.

When examining the relation between FMD and SRAUC, *Chapter 5* demonstrated that this relation is attenuated with age in healthy men and women. Interestingly, there was a significant main effect for age for blood pressure and BMI (Table 5.1). Despite this, blood pressure and BMI for the participants were well within the normal range for "healthy" (i.e. <140/90mmHg and <30kg/m^2, respectively). Additional regression analysis revealed that FMD is influenced by systolic blood pressure, but not diastolic blood pressure or BMI. Repeated correlation analysis in a subgroup with blood pressure <130/80mmHg (n=505) confirmed the age- and sex-specific differences in the FMD-SRAUC relationship. Therefore, it is unlikely that CVD risk factors explained the age-specific attenuation in the relation between FMD and SRAUC. As discussed in *Chapter 5*, the attenuation in

the FMD-SRAUC with age may be attributable to age-related changes in artery structure (Dinenno et al., 2000; Engelen et al., 2013), NO bioavailability (Taddei et al., 2001; Al-Shaer et al., 2006), and shear patterns (Credeur et al., 2009; Young et al., 2010; Padilla et al., 2011; Casey et al., 2012).

7.3.2.2 Shear Patterns or Fluctuations: What is More Important for Vascular Function?

Experimental research manipulating shear stress through exercise or heating has demonstrated improvements in FMD (Tinken et al., 2009; Greyling et al., 2015a; Naylor et al., 2011a; Brunt et al., 2016; Carter et al., 2014). However, these interventions resulted in increased mean shear rate, whilst the intermittent negative pressure-induced fluctuations in shear rate (*Chapter 6*) without altering mean shear rate compared to resting baseline levels. When examining shear patterns, the application of negative pressure (-40mmHg) caused significant increases and decreases in antegrade- and retrograde shear rate, respectively, compared to baseline. Then the release of negative pressure (i.e. back to atmospheric pressure) caused increased and decreased retrograde- and antegrade shear rate, respectively. Despite these fluctuations, average antegrade- and retrograde shear rate during the intervention was not different compared to resting baseline levels (Figure 6.3). Furthermore, regardless of the 'clamped' average shear rate, brachial artery FMD improved in the arm exposed to the intervention. This may be somewhat surprising, especially since increased antegrade- and retrograde shear rate induce opposing effects on FMD (Thijssen et al., 2009c; Schreuder et al., 2015; Johnson et al., 2012; Tinken et al., 2009) and evidence suggests that the endothelium may be responsive to changes in mean shear rate (Pyke et al., 2008; Tremblay et al., 2019). This raises the question related to the importance of each component of blood flow and shear rate (i.e. antegrade *versus* retrograde). FMD has been shown to improve following exercise/heating, consisting of different shear patterns (Tinken et al., 2009; Cheng et al., 2019), an effect of which was abolished when

antegrade shear rate was attenuated (Tinken et al., 2009). During the intermittent negative pressure intervention, the relative change for antegrade shear rate was ~20% (absolute change = $25s^{-1}$), whilst retrograde shear rate increased by ~60% (absolute change = $8s^{-1}$) compared to baseline. Since FMD improved despite the larger relative increase in retrograde shear rate, this data suggests that concomitant increases in antegrade shear rate may override the negative vascular effects associated with retrograde shear rate. It is also plausible that these findings were attributed to the absolute change in shear rate during the application of negative pressure (i.e. larger absolute change in antegrade compared to retrograde shear rate). Nonetheless, the findings presented in *Chapter 6* propose that increases in mean shear rate may not be necessary to improve endothelial function; rather, antegrade shear rate may be of greater importance.

Fluctuations in blood flow and shear rate may be related to engaging in regular low-intensity physical activity, particularly as a strategy to break up sedentary behaviour. An acute experiment by Thosar et al. (2014) found a decline in femoral artery FMD, but not brachial artery FMD following uninterrupted sitting, implying a local negative effect of sitting. Recent work has demonstrated that the prolonged sitting-induced decline in blood flow and shear stress, and subsequent vascular function can be prevented by breaking up sitting with physical activity breaks (Carter et al., 2018; Thosar et al., 2015) or even fidgeting (Morishima et al., 2016). In addition, intermittent negative pressure-induced fluctuations in blood flow and shear rate was shown to improve wound healing in patients with foot ulcers and disturbed blood flow (Sundby et al., 2016a; Sundby et al., 2017; Sundby et al., 2018b). These observations were largely stimulated by increased microvascular (skin) blood flow during the application of negative pressure (Sundby et al., 2016b), therefore improving oxygen/nutrient delivery to the wound (Tsai & Intaglietta, 1993b; Aalkjaer et al., 2011). Furthermore, *in vitro* and *in vivo* models have demonstrated

enhanced prostacyclin and NO release in response to pulsatile shear stress (Frangos et al., 1985; Nakano et al., 2000). Whilst greater abundance/availability of vasodilators may have explained the improvement in FMD observed in *Chapter 6*, these positive effects may be even further augmented in clinical populations. Given the observed effectiveness and tolerability of intermittent negative pressure from the above studies, further research is warranted into the feasibility of use as a treatment method, particularly in disabled/bed-bound individuals where physical activity is not a viable option. Taken together, in addition to the data presented in *Chapter 6*, these findings support the benefits of fluctuations in blood flow and shear rate, which also appears to positively affect multiple vascular beds.

7.3.3 Implications for the Performance of FMD

The large dataset presented in *Chapter 4* originated from FMD studies completed across six laboratories worldwide, with different investigators/sonographers, ultrasound machines and analysis software. Specific to the analysis software, five out of six laboratories used the same edge-detection and wall-tracking analysis software (BloodFlow Analysis), whilst one laboratory used FMD Studio (Quipu, SRL). To statistically account for this, multiple linear regression analysis revealed an insignificant regression coefficient of β=0.166%, which was used as a calibration factor to rescale individual FMD values obtained using FMD Studio. Importantly, all FMD data included in the large dataset were collected with strict adherence to expert-consensus guidelines (Corretti et al., 2002; Thijssen et al., 2011a) and the minimal calibration factor applied further reinforces that variability between FMD outcome is low when protocol guidelines are adhered to (Greyling et al., 2016a). Moreover, updated FMD guidelines (see *Chapter 3*; published in *European Heart Journal;* doi: 10.1093/eurheartj/ehz350) recommend research groups worldwide to adhere to protocol (and analysis) guidelines, in order to improve comparability of FMD outcomes between studies (Thijssen et al., 2019a). From

this, future FMD research should only be published if the guidelines have been met (and this should be monitored at the peer review stage), thereby maintaining the credibility of the FMD technique. Ultimately, estimation of reference values for brachial artery FMD enables improved interpretation of FMD outcome in addition to comparability between populations in a research setting.

7.4 Methodological Considerations: Strengths and Limitations

Key strengths specifically relate to the FMD methodology presented in this thesis. Firstly, despite the worldwide use of the FMD technique since the initial work by Celermajer et al. (1992), work presented in this thesis is the first study to construct reference values for brachial FMD in a large number of strictly healthy individuals. Importantly, all FMD data presented in *Chapters 4, 5 and 6* was obtained following strict adherence to expert-consensus guidelines for the performance and analysis of the FMD (Corretti et al., 2002; Thijssen et al., 2011a). Furthermore, as described above, *Chapter 4* demonstrated that despite different investigators and technology, variability in FMD outcome was low.

Despite this, the nature of the data collection for *Chapters 4 and 5* (i.e. retrospective design) provides a number of limitations to this thesis. Only a small number of studies included in the dataset collected data on physical activity and cardiorespiratory fitness, whilst no studies examined sex hormones or endothelial markers. Therefore, this information could not be included in the database. Furthermore, whilst multiple imputation was used to “fill in” missing data (e.g., blood pressure, BMI, baseline artery diameter), some datasets were missing critical (and easily obtainable) information related to CVD risk. More specifically, due to low availability, a sub-analysis was required to examine the relation of smoker status with FMD. Additionally, ethnicity was not included as a variable given the lack of available information (too little to warrant a sex-

specific sub-analysis). Therefore, it is imperative for researchers to collect (and report) more comprehensive participant information related to all CVD risk factors affecting FMD. For example, questionnaires can be used to assess physical activity, ethnicity and smoker status (plus number of cigarettes per day), whilst more experience (and funds) are required to assess blood markers and hormone levels. Nonetheless, these data would have complemented the data presented in this thesis to provide some mechanistic insight underlying our major findings.

7.5 Future Directions

Currently, the FMD technique is limited to use in a research setting, due to its non-invasive nature and susceptibility to respond to stimuli such as exercise and/or pharmacological interventions. As a result of *Chapter 4* of this thesis, the estimation of reference values for brachial artery FMD has provided greater clinical/prognostic meaning to the FMD. Therefore, with the new estimation of what is “normal” for healthy individuals, and given the association with atherosclerosis and CVD risk, the FMD technique may be ready for use in a clinical setting. Nonetheless, potential barriers to clinical use relates to qualified sonographers in addition to time constraints (i.e. 15mins rest, 9mins FMD protocol [1min baseline, 5mins cuff inflation, 3mins post]), and subject preparation (i.e. fasted state, avoid exercise and alcohol etc.).

Findings presented in this thesis have provided a stepping stone for future research within the field of cardiovascular physiology and contributed to the “hot topic” of sex differences. Females are largely underrepresented in human and animal physiological research (Beery & Zucker, 2011), which is also demonstrated by the difference in the number of females compared to males in the datasets presented in *Chapters 4 and 5*. The majority of physiological research is conducted in male participants, however “male”

data may not always translate to females, in the same way that an experiment in healthy participants may not apply to clinical populations. Potential explanations for the scarce physiological research in females may relate to the lack of knowledge and/or understanding of the impact of female-specific hormones; not only during a normal 28-day menstrual cycle, but also with the use of different hormone-based contraception methods and replacement therapy. Specific to the FMD (and other cardiovascular health markers), females are assessed during the first 7 days of the menstrual cycle when oestrogen is lowest, whilst those on hormone-based contraception or replacement therapy are generally excluded from cardiovascular studies. This immediately reduces the pool of eligible female subjects, therefore further highlighting the need for future research to improve understanding around the impact of sex hormones on cardiovascular health and function.

Another emerging theme from this thesis relates to novel findings presented in *Chapter 6*, highlighting the importance of fluctuations in shear rate for endothelial function. Given that the study was statistically underpowered, further work is warranted to better understand the potency of fluctuations in shear rate patterns on vascular function. More specifically, future research should endeavour to explore the underlying mediating mechanisms, in addition to the chronic effect of fluctuations in shear rate on vascular function in health and disease.

7.6 Conclusion

In summary, FMD data obtained with strict adherence to expert-consensus guidelines (Corretti et al., 2002; Thijssen et al., 2011a) allowed for the estimation of reference values for brachial artery FMD. These data demonstrated sex differences in the age-related decline in brachial artery FMD in healthy subjects, where female FMD starts

higher and declines at a faster rate. For the first time, *Chapter 4* has provided predictive equations for the estimation of brachial artery FMD in healthy individuals, and explored the relation with CVD risk factors. When exploring the role of total shear stress as the eliciting stimulus for FMD (i.e. SRAUC (Pyke & Tschakovsky, 2007)), the relation between FMD and SRAUC was stronger in women compared to men, and also declines with age (*Chapter 5*). Finally, the novel data presented in *Chapter 6* provided insight into the relevance of shear rate, specifically fluctuations in shear rate, as a stimulus for vascular health promotion.

Chapter 8

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Appendices

Appendix Table 1: Extended author list from participating centres (i.e. the TIFN Working Group), corresponding affiliations, and ultrasound devices and analysis software details.

Centre *(total n)*	Contributing Authors	Affiliations	Ultrasound devices used (probe MHz)	Analysis software
Liverpool John Moores University, United Kingdom *(n=1147)*	Sophie M. Holder[1], Sophie E. Carter[1,2], Áine Brislane[1,2], Kirsty A. Roberts[1], Joseph Maxwell[1], Sam N. Scott[1], Andrea Tryfonos[1], Benjamin J.R. Buckley[1], Tom G. Bailey[1], Scott Cocking[1,3], Daniel C. Perry[1,4], Girpreet K. Birk[1], Mark A. Black[1], Ellen A. Dawson[1], Nicola D. Hopkins[1], Helen Jones[1], Victoria S. Sprung[1,5], Daniel J. Cuthbertson[5], Christopher J.A. Pugh[1,6], Gemma D. Miller[1], Toni M. Tinken[1], Nicola Rowley[1], Stuart M. Ennis[6,7], Greg Atkinson[8,9], Nia C. Lewis[10].	[1]Research Institute for Sport and Exercise Science, Liverpool John Moores University, Liverpool, UK; [2]School of Sport and Exercise Science, York St John University, York, UK; [3]Athlete Health and Performance Research Centre, Aspetar Orthopaedic and Sports Medicine Hospital, Doha, Qatar; [4]Institute of Child Health, University of Liverpool, Alder Hey Children's NHS Foundation Trust, Liverpool, UK; [5]Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, UK; [6]Cardiff School of Sport, Cardiff Metropolitan University, Cardiff, UK; [7]University Hospitals Coventry and Warwickshire NHS Trust, Coventry, UK; [8]Health and Social Care Institute, School of Health and Social Care, Teesside University; [9]Department of Sleep Medicine, South Tees Hospitals NHS Foundation Trust, Middlesbrough, UK; [10]School of Health and Exercise Sciences, University of British Columbia Okanagan, Kelowna, BC V1V 1V7, Canada.	Terason T3000/3300 (10-12MHz); Aspen, Acuson (7.5-12 MHz)	Blood Flow Analysis
Radboud University Medical Center, The Netherlands *(n=1238)*	Dick H.J. Thijssen[1,2], Daria A. Shkredova[1,3], Anke van Mil[1], Maria T.E. Hopman[1], Tim H.A. Schreuder[1], Joost P.H. Seeger[1], Sean H.P.P. Roerink[1,4], Ralph R. Scholten[1], Arno Greyling[1,5], Martijn F.H. Maessen[1], Inge van den Munckhof[1], Jaap J. Brunnekreef[1], Margreet A. Wagenmakers[6], Nathasja van Leeuwen[1], Saloua E.I. Messaoudi[7],	[1]Radboud Institute for Health Sciences, Department of Physiology, Radboud University Medical Center, The Netherlands; [2]Research Institute for Sport and Exercise Science, Liverpool John Moores University, Liverpool, United Kingdom; [3]Centre for Heart, Lung, and Vascular Health, School of Health and Exercise Science, University of British Columbia, Kelowna, Canada; [4]Department of Medicine, Division of Endocrinology, Radboud University Medical Centre, The Netherlands; [5]Unilever Research & Development Vlaardingen, Vlaardingen, The Netherlands; [6]Department of Internal	Terason T3000 (10 MHz); Aspen, Acuson (7.5 MHz); VIVID E9 (15 MHz)	Blood Flow Analysis

	Nathalie M.M. Benda[1], Constantijn W. Wouters[7].	Medicine, Radboud University Medical Centre, The Netherlands; [7]Department of Pharmacology-Toxicology, Radboud University Medical Centre, The Netherlands.		
University of Pisa, Italy *(n=2118)*	Rosa Maria Bruno[1,2,3], Lorenzo Ghiadoni[1], Ferdinando Franzoni[1], Yvonne Plantinga[1], Fabio Galetta[1], Davide Carrara[1], Filippo Graziani[1], Edoardo Vitolo[1].	[1]Department of Clinical and Experimental Medicine, University of Pisa, Italy; [2]1INSERM, U970, Paris Cardiovascular Research Center (PARCC), Paris, France; [3]Paris Descartes University, Paris, France.	Esaote AU5 (7 MHz); MyLab25, Esaote (10MHz)	FMD Studio
University of the Sunshine Coast, Australia *(n=121)*	Christopher D. Askew[1,2], Annelise Meneses[1], Tom G. Bailey[1], Mark T. Windsor[1], Maria Perissiou[1], Timo Klein[1].	[1]VasoActive Research Group, School of Health and Sport Sciences, University of the Sunshine Coast, Sippy Downs, QLD 4556, Australia; [2]Sunshine Coast Health Institute, Sunshine Coast Hospital and Health Service, Birtinya, QLD 4575, Australia.	Terason T3000 (10 MHz)	Blood Flow Analysis
University of Queensland, Australia *(n=122)*	Tom G. Bailey[1], Jeff S. Coombes[1], Jenna L Taylor[1], Emily R. Cox[1], Trishan Gajanand[1].	[1]School of Human Movement and Nutrition Sciences, The University of Queensland, St Lucia, Brisbane, Queensland, Australia.	Terason T3000/3300 (10-12 MHz)	Blood Flow Analysis
University of Western Australia, Australia *(n=616)*	Daniel J. Green[1], Ceri L. Atkinson[1], Louise H. Naylor[1], Channa Marsh[1], Treya Long[1], Andrew Haynes[1], Michael J. Wheeler[1,2], Lauren McKeown[1], Andrew J. Maiorana[3,4], Michael M. Tymko[5], Phil N. Ainslie[5].	[1]School of Human Sciences (Exercise and Sports Science), The University of Western Australia, Crawley, Western Australia, 6009; [2]Baker Heart and Diabetes Institute, Melbourne, Victoria, Australia; [3]School of Physiotherapy and Exercise Science, Curtin University, Perth, Australia; [4]Allied Health Department & Advanced Heart Failure and Cardiac Transplant Service, Royal Perth Hospital, Perth, Australia; [5]Centre for Heart, Lung, and Vascular Health, School of Health and Exercise Science, University of British Columbia, Kelowna, Canada.	Terason T3000/T3200 (10-12MHz); Aspen, Acuson (10-12 MHz)	Blood Flow Analysis

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CURRENT OPINION

Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans

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Endothelial dysfunction is involved in the development of atherosclerosis, which precedes asymptomatic structural vascular alterations as well as clinical manifestations of cardiovascular disease (CVD). Endothelial function can be assessed non-invasively using the flow-mediated dilation (FMD) technique. Flow-mediated dilation represents an endothelium-dependent, largely nitric oxide (NO)-mediated dilatation of conduit arteries in response to an imposed increase in blood flow and shear stress. Flow-mediated dilation is affected by cardiovascular (CV) risk factors, relates to coronary artery endothelial function, and independently predicts CVD outcome. Accordingly, FMD is a tool for examining the pathophysiology of CVD and possibly identifying subjects at increased risk for future CV events. Moreover, it has merit in examining the acute and long-term impact of physiological and pharmacological interventions in humans. Despite concerns about its reproducibility, the available evidence shows that highly reliable FMD measurements can be achieved when specialized laboratories follow standardized protocols. For this purpose, updated expert consensus guidelines for the performance of FMD are presented, which are based on critical appraisal of novel technical approaches, development of analysis software, and studies exploring the physiological principles underlying the technique. Uniformity in FMD performance will (i) improve comparability between studies, (ii) contribute to construction of reference values, and (iii) offer an easy accessible and early marker of atherosclerosis that could complement clinical symptoms of structural arterial disease and facilitate early diagnosis and prediction of CVD outcomes.

Keywords Flow-mediated dilation • Methodology • Cardiovascular disease • Brachial artery • Vascular function

Introduction

Cardiovascular disease (CVD) remains the world's leading cause of morbidity and mortality.[1,2] Development of CVD starts in early childhood and progresses silently for many years,[3] ultimately resulting in angina, myocardial infarction, or ischaemic stroke. The endothelium plays a central role in the process of atherosclerosis from the first to the advanced stages. Due to its strategic anatomical position, it is highly responsive to detect various haemodynamic stimuli (e.g. shear stress, circumferential wall strain)[4] and regulates vascular tone, growth, adhesion, and coagulation in a endocrine-paracrine manner.[5] Not surprisingly, impairment of endothelial function precedes development of atherosclerosis, evident by thickening of the arterial wall and plaque formation.[6–9] Endothelial dysfunction is also linked to inflammatory changes within atherosclerotic plaques, leading to plaque vulnerability and, subsequently, increased risk for clinical events.[3,10] The intrinsic link between inflammation and endothelial dysfunction is well-established.[11,12]

The opinions expressed in this article are not necessarily those of the Editors of the *European Heart Journal* or of the European Society of Cardiology.

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Several (non-)invasive tools to examine endothelial function have been developed. These techniques provide insight into the pathophysiology of atherosclerosis and CVD, but may also predict cardiovascular (CV) events. The present article discusses flow-mediated dilation (FMD), which represents a popular and widely used non-invasive tool for examining peripheral artery endothelium-dependent dilation, originally introduced in 1992.[13] Briefly, this technique adopts ultrasound to examine changes in brachial artery diameter in response to ischaemia (typically 5 min), induced by inflating a blood pressure cuff distal from the imaged artery around the forearm to supra-systolic level. Importantly, substantial variation in performing the FMD is present, which impairs its reproducibility and comparison between studies. First, we will briefly discuss techniques utilized to assess endothelial (dys)function, and specifically focus on the clinical value of FMD. Subsequently, we will provide updated expert consensus guidelines for the performance of FMD, which are based on critical appraisal of novel technical advances, development of analysis software, and studies exploring physiological principles underlying the technique.

Vascular reactivity tests

Endothelial dysfunction, which is characterized by reduced nitric oxide (NO) availability,[14] was originally demonstrated by infusing acetylcholine in atherosclerotic coronary arteries.[15] Since this technique is invasive and not widely applicable, less invasive studies in the peripheral circulation have been then introduced (Supplementary material online, *Table*). A technique with methodological similarities to intra-coronary infusion involves venous plethysmography, typically of the forearm. After arterial cannulation, forearm blood flow changes after infusion of vasoactive substances modulating NO-release are measured. Alternatively, non-invasive techniques include peripheral arterial tonometry (PAT) and FMD, both measuring vasodilator responses after transient ischaemia. Whereas FMD examines macrovascular endothelial function of the brachial artery, PAT may represent a measure of finger microvascular function. Techniques have their specific advantages and disadvantages (Supplementary material online, *Table*), as extensively reviewed elsewhere.[14] Importantly, non-invasive techniques have been validated against coronary endothelial function, providing the pathophysiological basis for the predictive value of these tests for CVD.[16,17]

Clinical value of flow-mediated dilation

Association with cardiovascular disease prognosis

Impaired FMD has been associated with conditions predisposing to atherosclerosis and CVD, representing an early step in developing subclinical target organ damage and late clinical events.[18] Brachial FMD is associated with carotid intima-media thickness progression in a population free of CVD[7] and in hypertensive, postmenopausal women.[19] In a 3-year follow-up study in hypertensive patients, FMD-predicted target organ damage progression (including carotid intima-media thickness, pulse wave velocity, albuminuria, and left ventricular hypertrophy), even after adjustment for known risk factors.[20] Furthermore, impaired FMD represents an independent predictor of in-stent stenosis after single-vessel coronary interventions.[21]

Several studies have demonstrated the prognostic value of brachial artery FMD for CV events (*Table 1*).[22–25] Meta-analyses indicate a significant 8–13% lower risk of CV events per percent point increase in brachial artery FMD (e.g. from 5% to 6% dilation, *Figure 1*). This reduction was relevant both in high- and low-risk populations,[22,25] though appeared larger in patients with established CVD.[23,24] Except for one meta-analysis,[24] the overall estimated study quality did not significantly influence FMD predictive value.[22,23,25]

One meta-analysis of 14 studies assessed the influence of the site of the cuff position, which was either placed distal (i.e. forearm) or proximal (i.e. arm) from the imaged artery (*Table 1*).[26] This study found that the predictive capacity of the FMD using distal cuff position did not differ from using FMD with proximal cuff positioning. This was confirmed in a later meta-analysis that included a larger number of studies.[24]

Incremental value of flow-mediated dilation over traditional cardiovascular risk factors

Flow-mediated dilation has failed to demonstrate an added value to classical CV risk factors in terms of discrimination and net reclassification.[27] In the Multi-Ethnic Study of Atherosclerosis (MESA) cohort of intermediate-risk individuals, FMD did not increase discriminative power or net reclassification index of risk compared to the Framingham Risk Score.[28] However, the low reproducibility of the FMD in the MESA study, in which the intra-class correlation coefficient was 0.54[28] (worse than other studies in healthy volunteers[29,30] and CVD patients[30,31]), may have contributed to these negative findings. Alternatively, FMD may simply reflect compound risk burden that impacts upon vessel function and hence may not provide incremental risk prediction. A recent position paper by the *European Society of Cardiology* Working Group on Peripheral Circulation[32] concluded that FMD is principally a valuable research tool. Moreover, authors stated that, partly because poor standardization between laboratories and lack of guideline adherence, FMD is currently not recommended for the assessment of CV risk, with evidence grading remaining at III, in accordance with other guidelines.[33,34]

Clinical value of long-term changes in flow-mediated dilation

Longer-term improvement in FMD may have a prognostic implication.[35] Therefore, assessment of FMD in interventional trials could represent a surrogate endpoint, especially since FMD responds rapidly to therapies, allowing identification and selection of new drugs or bioactive substances.[36] The lack of improvement in FMD might identify 'non-responder' patients, who might be suitable for more intensive or new therapeutic approaches. For example, improvement in FMD following 6 months of antihypertensive therapy was associated with a more favourable prognosis for CV events in hypertensive postmenopausal women.[37] Furthermore, persistent impairment in FMD after optimized risk reduction therapy represents an independent predictor of CV events in patients with coronary artery disease.[38] Finally, a persistently impaired FMD, after optimized therapy in heart failure, independently predicted cardiac events.[39] These studies

Table 1 Summary of meta-analyses that included studies on predictive value of flow-mediated dilation on cardiovascular disease prognosis

Study	Number of studies (no CVD/CVD populations)	Number of subjects	Age range (years)	Follow-up (months)	Pooled CVD risk (RR[a]; 95% CI)
Inaba *et al.*[22]	15 (9/4)	5547	51–78	6–93	0.87; 0.83–0.91
Green, *et al.*[26]	14 (9/5)	8318	51–79	6–93	0.83; 0.78–0.88[b] 0.91; 0.87–0.69[c]
Ras *et al.*[23]	23 (15/8)	14753	46–79	12–96	0.92; 0.88–0.95
Xu *et al.*[25]	32 (19/13)	15191	47–73	6–115	0.90; 0.88–0.92
Matsuzawa *et al.*[24]	35 (22/13)	17280	46–79	6–115	0.88; 0.84–0.91

CI, confidence interval; CVD, cardiovascular disease; FMD, flow-mediated dilation; RR, relative risk.
[a]Per 1% higher FMD.
[b]Distal cuff occlusion.
[c]Proximal cuff occlusion.

support the potential use of repeated assessment of FMD, rather than a single measure, to predict future CV events.

Potential value of short-term changes in flow-mediated dilation

Many studies have examined short-term changes in FMD, ranging from a few hours, to several days, and up to months. In these randomized trials, FMD was selected as the primary outcome measure to investigate the potential protective impact of (non-)pharmacological therapies.[36,40–42] Increase in FMD in response to (non-)pharmacological substances may translate to long-term improvements in endothelial function and protection against atherosclerosis and/or CVD. The relative simplicity of the design and the ability to perform reproducible, repeated measurements explain the popularity of examining changes in FMD in these studies.[14]

In summary, a biomarker is only useful for CV risk prediction when it adds incremental information to traditional CV risk factors, being independently associated with outcomes, and also improving discrimination, calibration and net reclassification of risk, especially in those at intermediate risk.[43] Current evidence on these issues pertaining to FMD is incomplete. In part, this seems related to the diversity between studies in adherence to expert consensus guidelines. Moreover, reference values are yet to be established and are necessary for widespread applicability. Nonetheless, FMD has proven beneficial when examining (short-term) changes to evaluate the effects of (non-)pharmacological interventions.

Ultrasound assessment: technical requirements and common mistakes

Diameter and velocity assessment

Flow-mediated dilation is typically examined in the brachial artery (diameter 3–5 mm). Longitudinal images of the brachial artery are taken using high-resolution B-mode ultrasound, usually with an ultrasound probe of 7.5–12 MHz. Studies have also examined smaller (e.g. radial artery: diameter 1.5–3 mm) or larger sized arteries (e.g. superficial femoral artery: diameter 5–7 mm, popliteal artery: diameter 4–6 mm). Changes in resolution may be required to optimize B-mode images in arteries that lie at differing depths. A key challenge with B-mode imaging is to identify clear vascular boundaries, i.e. the double lines of Pignoli (*Figure 2*).[44–46] A reasonable approach involves obtaining an image characterized by a single echogenic layer, representing the brachial artery wall-lumen interface. Since diameter is a key determinant of FMD, the modality of measurement applied may influence results and, under all circumstances, should be kept the same throughout the experiment. Special attention should be paid to perform adequate scanning of the baseline diameter. More specifically, tangential scanning is a common error and results in underestimation of the true brachial artery diameter. Recent technological advances, which adopt an H-shaped probe capturing two short-axis and one long-axis for automatic probe position correction, may overcome this crucial limitation.[47]

Simultaneous live acquisition of the pulsed-wave Doppler velocity is recommended, given the importance of shear stress as the eliciting stimulus for dilation.[48–50] An important limitation of this approach is that the same transducer is employed to detect Doppler velocity and arterial diameter, which have competing requirements for optimal data acquisition. B-mode echoes are of greater intensity with perpendicular insonation of the ultrasound beam (90°), whereas optimal pulse-waved Doppler signals require parallel incidence with the direction of blood flow (ideally 0°, *Figure 2*).[51–53] Therefore, a compromise must be reached to uphold fundamental principles and assumptions for both modalities.[53] The error linked to incorrect estimation of insonation angle increases exponentially with angles greater than 60°.[51–53] Therefore, we recommend an insonation angle of <60–70° (which is the best achievable angle with standard ultrasound machines), which should be kept constant and reported in the manuscript.

Finally, the sample volume used for pulsed-wave Doppler should be considered. Since measurements settings of the width of the sample volume vary between laboratories and impact upon the analysis of the velocity signal (see Analysis of the Velocity Signal section), we recommend to maintain consistency in methods within studies, and in particular for repeated tests within subjects.

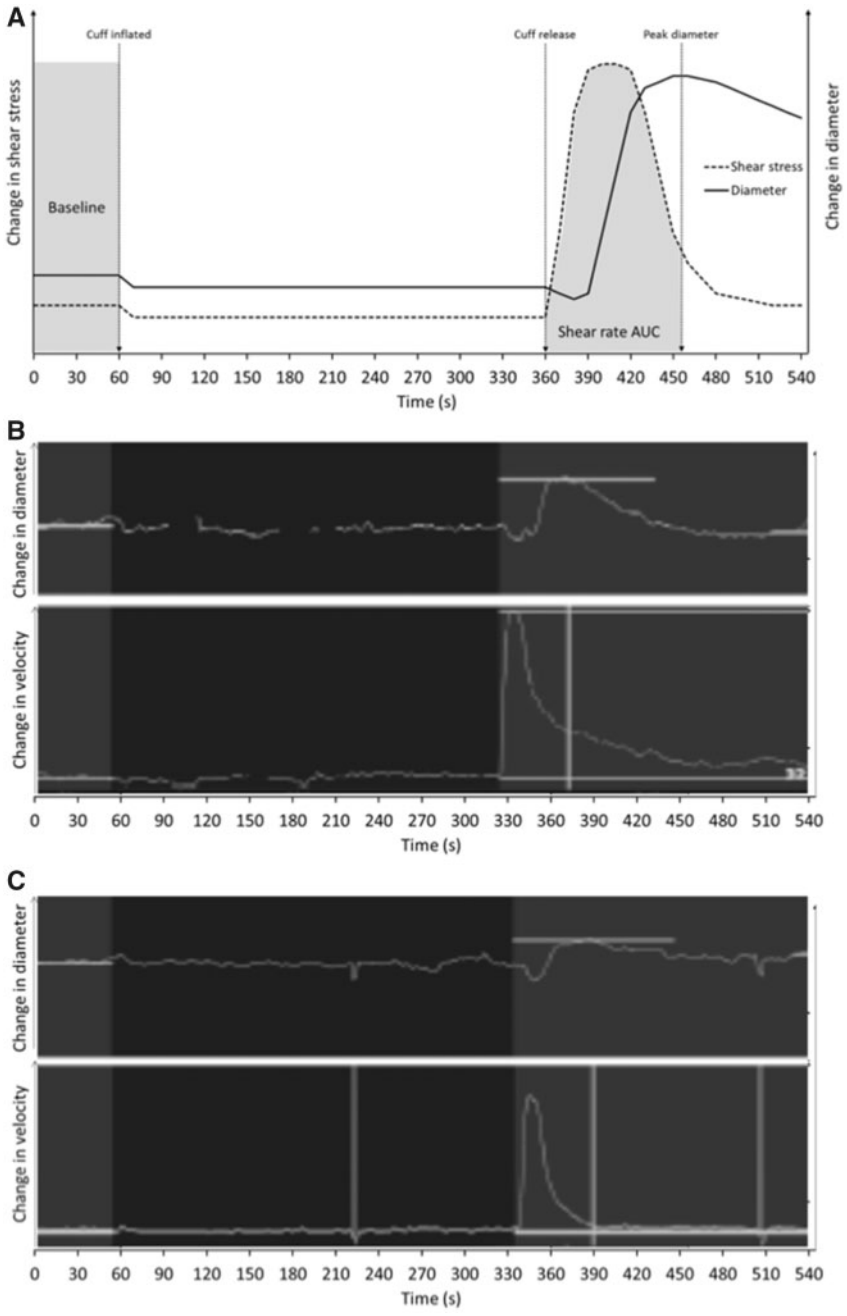


Figure 1 (*A*) Schematic presentation of diameter and shear stress (or rate) responses before and after a 5-min ischaemic stimulus applied distally to the forearm. (*B*) Representative diameter (upper panel) and velocity (lower panel) data from a healthy person before and after the 5-min ischaemic stimulus. (*C*) Representative diameter (upper panel) and velocity (lower panel) data from a person with endothelial dysfunction before and after the 5-min ischaemic stimulus.

Analysis of the velocity signal

Duplex ultrasound-derived velocity and diameter data are both required for calculating shear stress, the eliciting stimulus for artery dilation during FMD. Although the increase in shear stress is the physiological stimulus for dilation, most studies present shear *rate*, as it is assumed that blood viscosity does not differ between participants and/or groups.[50,54,55] Since no uniformity exists in the calculation of shear rate,[56] calculations must be described in the manuscript. Two different formulas have been suggested, according to the sample volume size and placement:

a. Shear rate = 8 × mean blood velocity/internal diameter; for large, centred sample volume;
b. Shear rate = 4 × mean blood velocity/internal diameter; for small, centred sample volume.

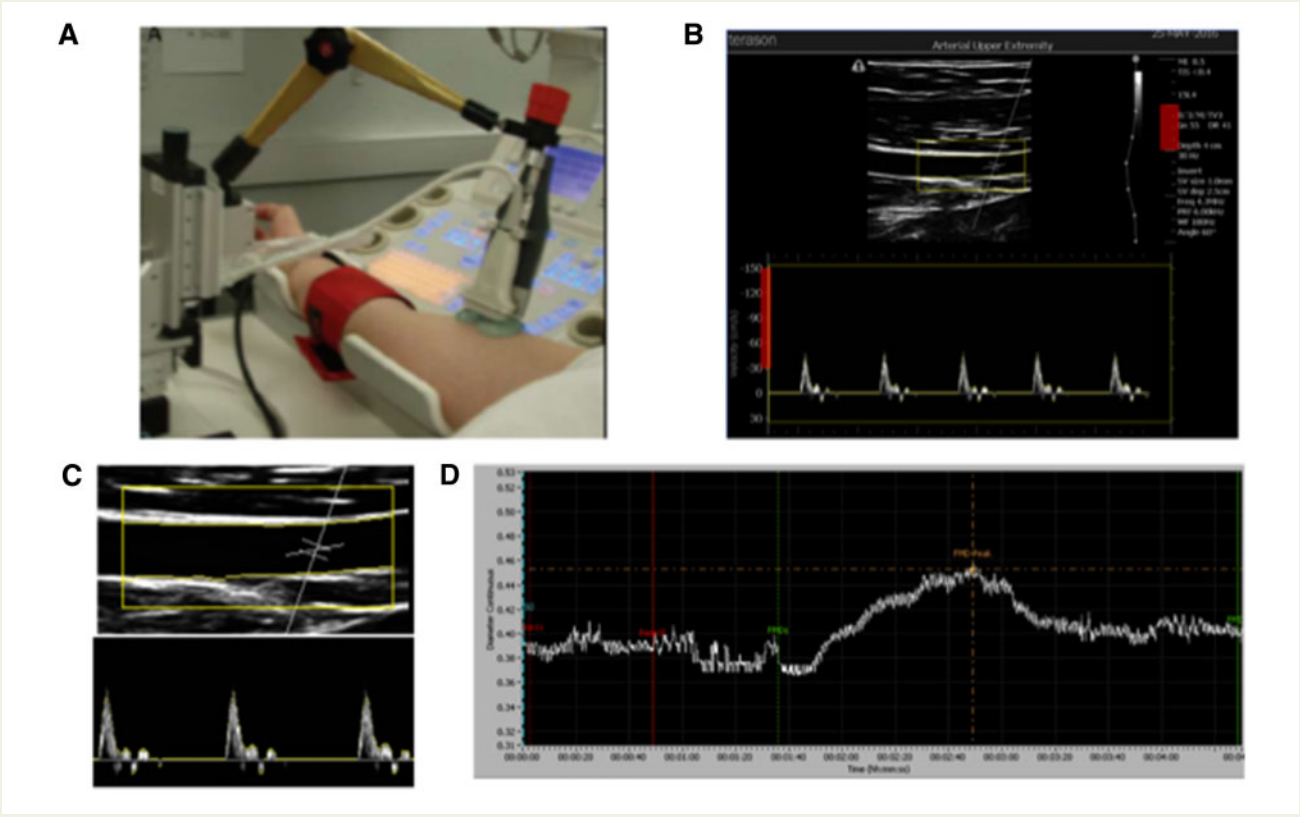


Figure 2 Experimental set up for the performance of the brachial artery flow-mediated dilation. (*A*) Position of the cuff and ultrasound probe placed on the upper arm. (*B*) Screen shot of a representative B-mode image of the diameter and Doppler blood flow velocity. (*C*) Wall tracking software to perform (semi)automated analysis. (*D*) Output generated by the analysis software to enable calculation of the flow-mediated dilation.

A clear description must also be provided for the calculation of velocity. Mean velocity can be estimated by taking half of the *peak velocity* (i.e. fastest moving blood cells in the centre of the vessel) or the *intensity weighted mean velocity* (i.e. mean velocity from all Doppler shifts across the vessel).[52] Although both methods seem valid, they cannot be used interchangeably.[57]

Maintaining the optimal image: probe-holder vs. hand-held

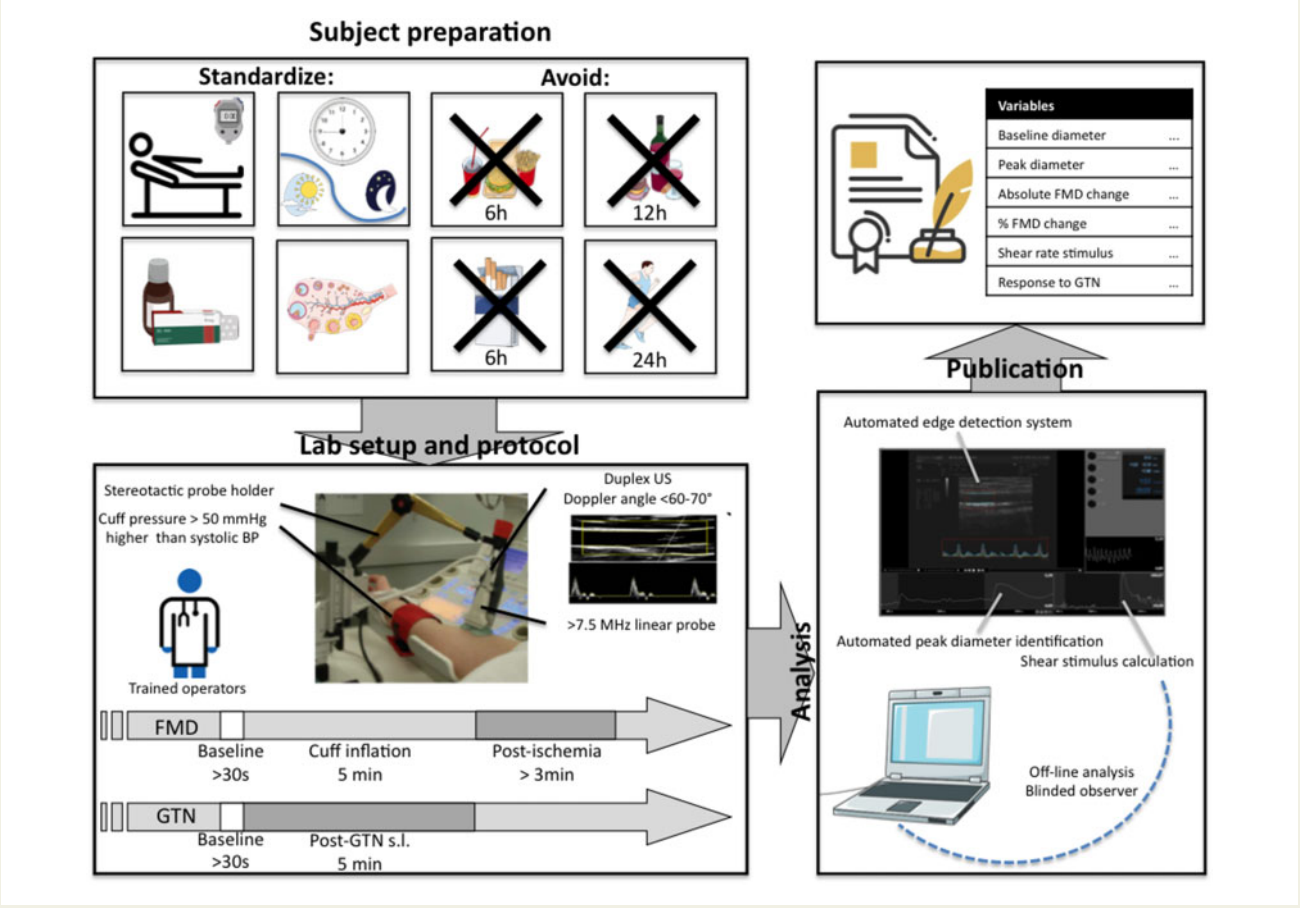
Another common mistake is inconsistency in scanning the same portion of the artery during repeated measurements, or throughout a single FMD. Therefore, anatomical landmarks should be identified and recorded and/or the distance between the elbow crease and ultrasound probe should be recorded (or photographed). The position of the subject (related to the arm and hand), but also cuff size and position (see Protocol: Cuff Position section) should be also recorded and standardized.[58]

To further ensure optimal image throughout the FMD-procedure, a probe-holding device can be useful.[29,58,59] A stereotactic adjustable probe-holding device allows adjustment of probe position during the test, ensuring that the same scan is maintained throughout the study.[18] Subject movement can also be compensated for when the probe hand-held, assuming an experienced sonographer. In a recent systematic review of studies that examined the reproducibility of the FMD, the use of a probe-holding device was associated with a higher FMD reproducibility.[60] However, another recent study pooling repeated measurements from different centres found that reproducibility is not affected by a probe-holder device, when experienced laboratories perform FMD following strict guidelines.[30] It cannot be excluded that other differences between laboratories may explain these findings. Nevertheless, a probe-holding device is a cheap, easily obtainable tool. Because it may facilitate FMD testing for inexperienced operators and increase accuracy, a probe-holder device is recommended, especially for less experienced laboratories.

Practical considerations, study protocol, and data analysis

Subject preparation and environmental influences

Subject preparation is important in the valid and reproducible assessment of the FMD, with many studies failing to control for these factors and/or failing to report on their procedures in sufficient detail (*Take home figure*). Several subject-related factors can influence FMD, including food, alcohol, smoking, supplements, drugs, physical activity, and mental stress.[61–64] While some factors directly impact stimulated NO-release, others, such as acute physical exercise,[62] modify baseline vasomotor tone (and therefore baseline diameter).[4] To minimize their effect, it is strongly recommended that



Take home figure Pictorial summary of the main recommendations for FMD evaluation in humans.

prior to FMD examination, subjects are fasted (>6 h), avoid exercise (>24 h), and refrain from caffeine, vitamin C, polyphenols, alcohol, and supplements known to affect the CV system for a consistent period of time (typically >12 h) (*Table 2*). Smokers must refrain from smoking for a standardized period (preferably >6 h). When examining patient groups taking drugs, we recommend waiting 4 times the half-life of the drug. If drug intake cannot be avoided, examination should be performed after a consistent and standardized time.[59] Since mental stress affects FMD,[64,66] testing must be performed in a quiet, temperature-controlled room after a standardized period of supine rest of >10–15 min. Acute intense mental stress can cause prolonged (up to 90 min) impairment in FMD,[67] but its confounding effect can be hardly controlled or eliminated. Premenopausal women should be examined in a standardized phase of the menstrual cycle, since hormonal changes can affect FMD.[68] Vascular function in humans, including that assessed by FMD,[69] demonstrates a diurnal variation. This implies that the time of the day of FMD should be standardized and reported, especially when performing repeated measures. Other environmental factors that may affect FMD are outdoor temperature,[70] seasonality,[71] and air pollution.[72] However, because of practical reasons, these factors should not be considered as limiting when ideal conditions cannot be achieved.

Study protocol

Protocol: cuff position

Placement of the occlusion cuff importantly alters the magnitude,[73] duration,[73,74] nature,[75] and possibly the clinical relevance[26] of the dilator response. Originally, Celermajer *et al.*[13] examined brachial artery FMD with the occlusion cuff placed around the forearm, distal to the ultrasound probe (*Take home figure*). Standardizing cuff position is of crucial importance for valid comparisons between studies, since occlusion of a smaller volume (i.e. placement towards the wrist) or larger volume of tissue (i.e. cuff occlusion around the upper arm) leads to either a markedly attenuated diameter[58] or an enhanced[73,76] shear stress response.

Some have supported the placement of the occlusion cuff above the imaged artery,[74] with the main argument that the larger dilator response[77] achieved by occluding a larger area may improve FMD discriminative power. One analysis found no significant differences in prognostic value between studies that adopted distal vs. proximal cuff position.[26] Furthermore, some limitations of proximal cuff inflation must be considered. First, scanning the brachial artery during this procedure is challenging, since the artery can collapse and/or tissue movements can worsen image quality. Secondly, the dilator response with proximal cuff position is largely mediated through vasoactive substances other than NO.[61] A meta-analysis[75] found that the dilation in response to distal cuff occlusion is ~70% NO-mediated, whereas proximal cuff

Table 2 Recommendations for flow-mediated dilation assessment to examine a largely nitric oxide-mediated, endothelium-dependent vasodilation of a conduit artery

Methodological and technical guidelines	Required
Subject preparation	
Rest in a quiet, preferably darkened room for a period of 10–15 min prior to assessment.	1
Supine posture (i.e. the imaged artery should be around heart level).	1
Testing times must be standardized to avoid diurnal variation, and for multiple tests, all are to be conducted at a similar time of day	1
Subjects must be fasted for ≥6 h	2
Avoid exercise for ≥24 h, alcohol or food/drinks that contain caffeine or are rich in polyphenols for ≥12 h	2
No smoking or any tobacco consumption prior to measurement (>6 h)	2
Careful history should be taken regarding the use/timing of any drugs. Drug withdrawal may be required, but this depends on the protocol and is not always feasible (in patients) or required	2
Premenopausal women should be assessed in standardized part of the menstrual cycle (especially when performing repeated measures) or menstrual phase should be annotated.	2
Protocol	
Baseline diameter must be examined before cuff inflation for a period of >30 s	1
Present absolute baseline diameter in Results section	1
Cuff must be placed distal to the imaged artery, with cuff pressure exceeding systolic pressure with >50 mmHg and inflated for 5 min	1
Post-deflation diameter should be monitored continuously from deflation for ≥3 min	1
Endothelium-independent vasodilation should be tested and GTN dose should be reported	1
Sublingual administration of 25 μg is suggested	3
Technique	
Continuous measurement of velocity and diameter using simultaneous live duplex ultrasound	1
B-mode images with a linear probe of ≥7.5 MHz should be used (and report probe details). The highest MHz available should be used, given tissue depth considerations	1
Blood velocity assessed using an insonation equal to 60–70° or less	1
Use the same angle for blood flow velocity within a study and study group (and report angle)	1
Analysis	
Use continuous edge-detection and wall tracking software	1
Automated mathematical algorithms should be used to calculate the peak diameter	1
Present the baseline diameter	1
Present the FMD-response in absolute (in mm) and relative (in %) change	1
Present the relevant shear rate stimulus (area-under-the-curve or peak shear)	2
Off-line analysis is performed by a blinded observer	3
Operator-dependent factors	
Sufficient training of sonographer (recommended levels of reproducibility: <2% coefficient of variation for diameter, <15% coefficient of variation for FMD in consecutive scans)	1
Maintain sonographer experience	1
A stereotactic probe-holder is recommended for less experienced laboratories	2

Based on previous work,[65] the right column indicates the level of requirement for specific recommendation: mandatory (1), highly recommended (2), and recommended when possible (3).

placement is only ~30% NO-mediated. These differences between studies are confirmed by direct comparisons of distal vs. proximal cuff position in the same individual.[74] This is mechanistically important, because several studies specifically aim to study the NO-dependent pathway, given its established importance in the process of atherosclerosis. Thus, strict standardization of distal cuff occlusion (i.e. below the imaged artery) is recommended to ensure maximal dependence of the dilator response on endothelium-derived NO.

Protocol: baseline diameter

The FMD-response is typically expressed as the relative change in post-hyperaemia diameter from baseline. As a direct consequence and limitation, the FMD is a function of the degree of responsiveness to stimuli, but also of baseline vasomotor tone and structural remodelling. Correct assessment of baseline diameter is therefore of crucial importance and implies the removal of potential factors influencing both baseline diameter and vasomotor tone (see Subject Preparation and Environmental Influences section), but also practical (Diameter and Velocity Assessment section + this section) and statistical procedures (Shear Stimulus: Normalization for flow-mediated dilation Stimulus section + this section) linked to baseline diameter.

Most studies use the pre-inflation diameter to calculate the FMD, in line with the classic approach[13] and previous FMD-guidelines.[58,59,61] It is recommended that pre-inflation diameter should be

recorded for 1 min, with a minimum of 30 s.[61] The end-of-ischaemia diameter has been also proposed as the baseline diameter for FMD calculation. However, 'low-flow' state during distal cuff occlusion may lead to artery constriction, though this phenomenon appears to be evident mostly in the radial[78–80] rather than in the brachial artery.[49,81–83] Conversely, one study showed that brachial artery diameter during cuff inflation was significantly larger than that assessed pre-inflation, leading to a lower FMD, an effect not present in older groups.[83] Based on these between-group differences, we recommend using pre-occlusion diameters as the baseline value so that the differential impacts of cuff occlusion on the baseline artery diameter are avoided.

Because FMD is defined as increase in diameter after occlusion, baseline diameter is strongly and inversely related with FMD.[13,84,85] This relation has several important implications for comparing groups that differ in baseline diameter (e.g. age, CVD),[86,87] and for interpretation of studies with interventions that may affect resting diameter (e.g. exercise training).[4] Atkinson *et al.*[88] found that the FMD% does not accurately scale for inter-individual differences in baseline diameter but overestimated endothelial function for low baseline diameters and *vice versa*, thus proposed to adopt allometric scaling, a statistical method to account for the relationship between baseline and peak diameter.[84] The use of allometric scaling, however, is limited by its inability to adjust FMD values at an individual level.

Protocol: duration and magnitude of cuff occlusion

The original 5-min duration of cuff occlusion remains the most frequently used protocol. Shorter periods lead to negligible dilation,[89] whilst longer periods lead to larger blood flow and diameter responses, but may be less tolerable for volunteers.[73] Since the nature of dilation changes with longer periods of occlusion,[76] we recommend using a 5-min occlusion period.

Whilst a majority of studies use a pre-defined cuff occlusion pressure (typically between 200 and 300 mmHg), it is important that cuff pressure should exceed >50 mmHg above systolic pressure to prevent arterial inflow. In our experience, the procedure is generally well tolerated and drop-out due to discomfort is unusual, though no studies are available in literature specifically dealing with this aspect. A common-sense recommendation to minimize subject discomfort is to use a smaller-sized (5 cm), paediatric cuff instead of a standard (12 cm) cuff. In addition, providing clear information about the study protocol and FMD-procedure is essential.

Protocol: post-deflation measurements

In the first studies using the FMD approach, peak diameter was examined using static frames at or around 60-s post-deflation.[13] During the past decade, several studies found that this approach (or using pre-determined time-windows) can significantly underestimate the true peak dilation.[90] More importantly, the timing of the peak dilation may differ between groups[90] or after interventions.[90–93] Therefore, to ensure successful capture of the true peak brachial artery diameter, guidelines support continuous examination up to 180-s post-deflation.

Endothelial function is increasingly examined in conduit arteries other than the brachial artery. However, the nature of the diameter responses in other arteries may differ from that in the brachial artery. For example, the content of endothelial NO synthase is heterogeneous throughout the arterial tree.[94] This may impact upon the relative contribution of NO to the dilator response. In addition, lower limb arteries show a significantly delayed peak dilation compared to those in the arms.[95] This means that the 3-min post-deflation time window may not be long enough for all arteries. These potential differences must be considered when performing and interpreting the FMD in other conduit arteries.

Identification of the peak diameter

Early studies applied electrocardiogram (ECG)-based-gated analysis to identify peak diameter.[59] This approach assesses the diameter at the onset of R-waves (i.e. end-diastole), which limits the influence of arterial compliance on the assessment of the diameter. In the past decade, driven by the technical possibility and recognition of its relevance, studies typically adopted continuous data analysis of the diameter across the cardiac cycle. Continuous data analysis shows good agreement with ECG-gated analysis[96,97] and is more time-efficient.[97] The accuracy and availability of this procedure makes ECG-gating no longer mandatory for determining peak artery diameter.

Shear stimulus

Shear stimulus: importance for flow-mediated dilation

Although the terminology of FMD suggests that dilation is flow mediated, physiological work demonstrates that it is rather mediated through the post-deflation increase in shear stress.[48] Studies that adopted within-subject manipulation of the post-deflation shear stress stimulus revealed that the relation between shear stress and dilation is dose-dependent.[48,49,98,99] This fits with landmark studies[100,101] demonstrating the importance of shear stress in mediating endothelium-dependent dilation. Pyke and Tschakovsky[49] suggested that the total, rather than peak shear stress, is more important in mediating conduit artery dilation.[49] This finding was later confirmed by others.[50] These studies show physiological and mechanistic basis for the continuous recording of both conduit artery diameter and velocity (*Figure 1*).

Shear stimulus: normalization for flow-mediated dilation stimulus

Based on the importance of shear stress as the eliciting stimulus, and also the assumption that within-/between-subject variation in FMD-responses relate to the magnitude of reactive hyperaemia, studies have used different approaches to account for the shear stress stimulus. An early approach involved 'simple' ratio normalization by dividing FMD by shear rate stimulus.[50,82,102] However, this approach tends to violate important statistical assumptions, being (i) the relationship between both parameters is linear, (ii) the intercept for the regression slope of this relationship is zero, (iii) data (including residuals) are normally distributed, (iv) variances are similar between groups, and (v) the ratio does not lead to spurious correlations with other variables.[103] Whilst acceptable-to-good relation between the shear rate stimulus and FMD is present *within* subjects,[48–50,89,99,104] studies examining this relation *between* subjects found a weak[95] or absent[105] relation. Whilst this does not invalidate the role of shear stress as the dilator stimulus, it indicates that FMD variability cannot be simply controlled for by ratio normalization.[103]

Studies have also explored other strategies to statistically correct the FMD-response for the shear stress stimulus, such as including shear rate as a covariate in an analysis of covariance.[103,106,107] Covariate in an analysis of covariance results, however, could be misleading if the covariate (i.e. shear rate), is related to the independent and/or outcome variable (e.g. age, sex, baseline diameter, and FMD). However, since calculation of shear rate importantly depends on baseline diameter, shear rate normalization may not be needed if the FMD is scaled properly to baseline diameter using the previously mentioned allometric scaling.[108]

Taken together, it is currently not clear which statistical strategy is preferred to account for the eliciting shear rate stimulus in the FMD response. Ratio-normalization seems statistically flawed, whilst too many questions remain present around validity, practicality and potential clinical importance of statistical correction for the shear rate stimulus. It is recommended to report the relevant shear rate stimulus, i.e. shear area-under-the-curve up to the peak diameter. Reporting the shear stress stimulus may also be clinically relevant because the post-occlusion hyperaemia is strongly related to CV risk, and may have prognostic value for future CV events.[109–112]

Analysis: Blinding, edge-detection, and wall-tracking

Early FMD research relied on a manual approach to assess diameter change through visual inspection and calipers placement.[13,113] This method is highly operator-dependent, carries risk of observer error and is time-consuming.[45,114–117] Recent years, software systems for automatic diameter measurement have been developed. This represents a time-efficient approach and has limited operator-related errors and bias. More importantly, intra-observer variation is significantly lower with automated analysis compared to the classic manual technique.[45,46,114,116–118]

Automatic wall tracking algorithms belong to two main categories according to the input signal: radiofrequency (RF) backscattering or grey scale speckle pixel intensities. In general, RF-based algorithms are mono-dimensional, with raw data processed in a single vertical line of view, while speckle tracking is applied on large bi-dimensional regions of interest. Previously, the RF-system was considered more accurate due to higher axial spatial resolution, but this comes at the cost of assessing a single arterial diameter site (analogous to the use of a single caliper dimension). Pixel density systems allow for multiple diameter assessments within a given frame, whilst recent speckle tracking systems have also overcome the spatial resolution limitation.[119]

Adoption of automatic software overcomes methodological issues linked to the use of manual calipers. Since maximal brachial vasodilation is 0.1–0.4 mm and the typical resolving power of manual calipers of 0.1 mm, the manual approach is likely to introduce significant within- and between-observer error. Manual measurements also have a low number of samples per time-frame, whilst a more detailed time-course of changes in diameter is desirable, especially to identify the true peak diameter.[90] Among potential advantages offered by automatic systems, real-time analysis allows for instantaneous feedback on scan quality and rapid adjustments in response to patient/probe movements. This real-time feedback may help the sonographer to optimize image quality. Another potential advantage is to immediately identify technical failure of the measurement, with the possibility to repeat the FMD after an adequate resting time.

An additional advantage of automated systems is their ability to use both ECG-gated and non-gated ultrasound images without affecting the FMD results.[96] This can help to keep the experimental setup as simple as possible, for example, by adopting a cheaper entry-level ultrasound device without ECG synchronization capability. Software systems for automatic FMD evaluation should be usable and user-friendly, but also should provide options to view and record different parameters (i.e. shear rate). Furthermore, the high throughput of data, ease of use and other technical features (e.g. real-time feedback, overlay of edge detector output with B-Mode) may help adequate training of the operator. Finally, any new automatic system must be validated in appropriate populations before introducing its use in laboratories.[45,120]

Summary and future directions

In conclusion, strong evidence supports the following methodologies: placement of a lower cuff occlusion and use of continuous diameter monitoring by automatic edge-detections systems. Furthermore, the general study protocol, subject preparation and lab requirements are well defined. It remains uncertain whether and how to correct FMD for the shear stimulus and/or baseline diameter. The pathophysiological significance and clinical relevance of additional parameters, such as flow-mediated constriction and reactive hyperaemia as a marker of microcirculatory function, are still matter of debate and require further investigation.

Reproducibility of flow-mediated dilation measurements

Several studies have examined FMD reproducibility. However, these have been performed with widely varying adherence to FMD-guidelines. After pooling the results of seven centres that strictly followed FMD-guidelines, including optimal training of operators and monitoring, Ghiadoni *et al.*[29] found that FMD in healthy volunteers is highly reproducible among centres (coefficient of variation 11.6–16.1%). Data from the dal-VESSEL trial also showed reproducible results in 19 laboratories after standardized training in patients with CVD (coefficient of variation 15.8–17.5%).[31] More recently, Greyling *et al.*[121] designed a tool to evaluate adherence to FMD expert consensus guidelines. Subsequently, a meta-analysis was performed to assess the relation between degree of adherence and reproducibility of the FMD.[60] Not surprisingly, stricter adherence to guidelines was related with markedly less measurement error in brachial artery FMD. This work highlights that strictly following contemporary guidelines leads to highly reproducible results.

Factors influencing reproducibility: group and methodology differences

Guidelines for FMD measurement cannot cover and eliminate all sources of measurement variations and error. Understanding of these factors is important for designing studies on FMD (e.g. sample size calculations). van Mil *et al.*[30] recently performed analysis of 672 repeated FMD measurements that were collected after strictly following contemporary guidelines. High reproducibility was found in

healthy subjects (9.3%), whilst regression models several factors that contributed to variations in reproducibility (see below).

Subject characteristics

Subjects with older age, hypertension, or dyslipidaemia show a larger variability in FMD.[29–31] This supports earlier findings in that CV risk factors impair FMD reproducibility.[65] An explanation for the larger variation in high-risk subjects may relate to the lower baseline FMD% typically observed in these populations.[58,122,123] Indeed, van Mil *et al.*[65] demonstrated that a lower FMD% explained part of the higher variability in FMD with older age and dyslipidaemia. Differences in vascular structure and compliance may also contribute to this higher variability[124] and finally, not all subjects may be equally susceptible to the effects of CV risk factors at the endothelial level.

Methodological characteristics (time between measurements)

Longer time periods between subsequent FMD measurements leads to larger variation.[29,30] Nonetheless, when strictly following guidelines and controlling for potential physiological factors,[29] it is possible to detect FMD changes, induced by interventions, over time with a relatively low sample size. Current data on within- and between-subject variability justify the use of FMD as an outcome measure in short-to-medium-term studies to evaluate (non)pharmacological interventions in humans.

Methodological factors (lab experience)

Possibly the most important methodological source of variability in the FMD is proper operator training. Guidance and training by expert colleagues seems crucial, since lower variability is present in more experienced laboratories.[30] Although the need of experience in scanning is mentioned by previous guidelines, [58,59,61] it is difficult to quantify 'proper training'. No study specifically aimed at quantifying the appropriate duration of the training, like what was done for carotid intima-media thickness.[125] Corretti *et al.*[59] suggested that >100 scans/year are required to maintain competency. However, there was no mention of the training required before performing FMD measurements. Two independent consortia adopted a formal training for sonographers: sonographers were qualified for measurements when the CV for repeated scans were <2% for brachial artery diameter and <15% for FMD.[29,31] Since FMD variability seems larger in individuals with CV risk,[29,31] operator training might be longer depending on the study population enrolled. Both aforementioned studies showed excellent reproducibility among centres, suggesting that specialized training is feasible and successful. We therefore recommend that all studies on FMD should report details on preparation and training of the sonographers, along with the observed variability in their FMD measurements.

Endothelium-independent dilation

Pathophysiology and clinical relevance

The degree of FMD can also be influenced by the functionality of vascular smooth muscle (VSM) cells.[126] Any functional defect in VSM cells reduces their capacity to respond to NO, whilst a compensatory increased VSM response may be present in endothelial dysfunction.[127,128] In any case, it is recommended that the studies on endothelium-dependent vasodilation also determine the extent of any coexistent endothelium-independent dysfunction. Although this recommendation was already present in previous FMD-guidelines,[59] a standardized protocol has not been clearly defined yet.

Clinical determinants of VSM function are not fully understood. In a large Japanese cohort, brachial artery dilation to sublingual glyceryl trinitrate (GTN) was correlated with most classical CV risk factors and represented an independent determinant of FMD.[129] Conversely, a meta-analysis reported micro-, but not macrovascular, impairment of VSM in Type 2 diabetic patients.[130] Since the brachial artery was studied in only 12 out of the 31 studies, this subgroup analysis is likely underpowered. Furthermore, some studies found a relation between brachial artery GTN-responses and lack of nocturnal blood pressure fall,[131] coronary artery calcium,[132] and microalbuminuria.[133] Although GTN-responses show a relationship with Framingham Risk score,[129] only few prospective studies investigated the relation between this response and CVD events.[134,135] Thus, the prognostic value of the GTN-response remains mostly inconclusive.[134,135] Finally, the doses typically used in these studies induce near-maximal dilation,[73] hence the GTN-responses may be a structural index rather than a functional one.

Dose, technique, and administration of exogenous nitrates

Many research centres use sublingual GTN 400 μg, the lowest marketed dose in many countries. Administration of GTN via a sublingual spray leads to a larger and faster response than by tablet administration.[136] Lower dosages (25 μg) of GTN were later introduced[137] and recommended in the 2005 ESH-statement.[138] The rationale for lower dosages is the induction of less extreme dilatation, a faster return to baseline diameter (~20 min), and lower risk of side effects. In healthy volunteers, 8–35 μg GTN is estimated to induce a 4–10% dilation, without changes in blood pressure and heart rate.[129,139] Furthermore, 25-μg GTN did not cause any significant increase in sympathetic signalling directed to muscle vasculature, evaluated by microneurography.[140] It has been suggested that higher GTN doses might be useful to assess maximum vasodilating capacity, thus providing information about structural alterations. However, maximal vasodilation cannot conceivably be obtained in these conditions because of counter-regulatory sympathetic vasoconstriction induced by the high doses of GTN. Although a low-dose GTN is recommended, this approach may not always be feasible, since low-dose GTN for oral use is not commercially available for most countries.

Glyceryl trinitrate should be administered >10 min after FMD testing to ensure return to baseline diameter. Furthermore, continuous diameter monitoring is recommended, because the timing of peak dilatation varies between subjects.[141] The peak vasodilation response to GTN usually occurs between 4 and 5 min, both with high-[141,142] and low-GTN doses.[137] Therefore, monitoring should be performed >5 min after GTN administration.

Conclusions

Flow-mediated dilation provides valuable and independent prognostic information. Unfortunately, different methodological approaches

importantly limit its validity, comparability, and its potential use as a clinical and physiological research tool. Indeed, adherence to guidelines and appropriate operator training improve FMD variability. Therefore, performing and reporting FMD according to state-of-the-art guidelines (see *Table 2*) is crucial to ensure valid conclusions and clinical evaluation. The application of state-of-the art methodology allows examining whether the FMD correctly reclassifies individuals, adding incremental information to traditional CV risk factors. Finally, strict adherence to guidelines will also contribute to reference values, which will further improve the clinical applicability of FMD.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Conflict of interest: F.F. and L.G. reported to be shareholder of Quipu s.r.l as relevant financial activities outside the submitted work. The other authors have nothing to disclose.

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