THE IMPACT OF ARTERIAL CATHETERIZATION ON VASCULAR FUNCTION IN HEALTHY SUBJECTS AND PATIENTS WITH CORONARY ARTERY DISEASE

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ABBREVATIONS

ACh	Acetylcholine
ACS	Acute coronary syndrome
ASVD	Arteriosclerotic vascular disease
AUC	Area under the curve
AUC+ve	Area under the curve (anterograde) – refers to blood flow
AUC-ve	Area under the curve (retrograde) – refers to blood flow
PBMC	Peripheral blood mononuclear cell
BH_4	Tetrahydrobiopterin
BMI	Body mass index
BMS	Bare-metal stents
BP	Blood pressure
CABG	Coronary artery by-pass graft
CAD	Coronary artery disease
CATH	Catheterized (arm)
CON	Control (arm)
CR	Cardiac rehabilitation
C-RP	C-reactive protein
CVA	Cerebrovascular accident
CVD	Cardiovascular disease
DAPI	4-6-diamidino-2-phenylindole hydrochloride
DBP	Diastolic blood pressure
DES	Drug-eluting stents
DPBS	Dulbecco's Phosphate Buffered Saline
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cells

ET-1	Endothelin-1
FMD	Flow-mediated dilation
GTN	Sublingual-glyceryl-trinitrate spray
HE	Handgrip exercise
HIE	High intensity exercise
HIIT	High intensity interval training
HR	Heart rate
HUVEC	Human umbilical vein endothelial cell
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IMT	Intima media thickness
LDL	Low-density lipoprotein
LHCH	Liverpool Heart and Chest Hospital
LJMU	Liverpool John Moores University
LSD	Least significant difference
MAP	Mean blood pressure
MIE	Moderate intensity exercise
MVC	Maximal voluntary contraction
NAD(P)Hox	Nicotinamide adenine dinucleotide phosphate oxidase
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	National health system (for UK)
NO	Nitric oxide
NOX2	Nicotinamide adenine dinucleotide phosphate subunit 2
Non-obCAD	Nonobstructive coronary artery disease
NT	Nitrotyrosine
$O_{2^{\cdot}}^{-}$	Superoxide
obCAD	Obstructive coronary artery disease
p16	Cyclin-dependent kinase inhibitor 2A

p21	Cyclin-dependent kinase inhibitor 1
p53	Tumor protein p53
PCI	Percutaneous coronary intervention (angioplasty)
PDGF	Platelet-derived growth factor
PECAM-1	Platelet endothelial cell adhesion molecule 1
peNOS Ser^{1177}	Phospho-eNOS at Ser^{1177}
PGI_2	Prostacyclin
pPECAM-1 Tyr^{713}	Phospho-PECAM-1 at Tyr^{713}
PTCA	Percutaneous transluminal coronary angiography
RA	Radial artery
REC	Research Ethics Committee
ROS	Reactive oxidative species
RPE	Rating of perceived exertion
SASP	Senescence-associated secretory phenotype
SBP	Systolic blood pressure
SD	Standard deviation
sNOX2-dp	soluble NOX2 derived peptide
SR	Shear rate
SRAUC	Shear rate area under the curve
SRAUC+ve	Anterograde shear rate area under the curve
SRAUC-ve	Retrograde shear rate area under the curve
TBARS	Thiobarbituric acid reactive substances
TNF-a	Tumor necrosis factor alpha
TXA_2	Thromboxane
VE-Cadherin	Vascular endothelial cadherin
VEGFR-2	Vascular endothelial growth factor receptor 2
VO_{2peak}	Peak oxygen consumption
VSMC	Vascular smooth muscle cell
Vwf	Von willebrand factor

ABSTRACT

Coronary artery disease (CAD) is the leading cause of global death. Diagnosis and treatment for CAD often involves angiography and/or angioplasty. However, the radial artery catheterization required during both procedures may result in acute artery dysfunction/damage, mainly due to endothelial disruption. Whilst exercise training typically enhances endothelial function, and is therefore generally recommended for CAD patients, animal studies indicated paradoxical exercise-induced vasoconstriction post-catheterization. It is not currently known if there is an acute period when exercise is detrimental due to catheter-induced damage.

Chapter 4 has demonstrated that catheterization in CAD patients results in impaired flow-mediated dilation (FMD), but that arterial responses to handgrip exercise (HE) are preserved 1 week post-catheterization. This finding suggests that the impact of endothelial disruption may be stimulus specific, with redundant mechanisms likely to preserve exercise-mediated vasodilator responses in the face of catheterization-mediated damage. **Chapter 6** showed that catheterization in young healthy males with a fully functional endothelium resulted in reduced FMD, including completely abolished FMD (≤0%) in approximately 1/5th of participants. This exaggerated response in healthy subjects, compared to CAD patients, raises the question of whether individuals with *a priori* endothelial dysfunction are more or less susceptible to catheterization-induced arterial risk. Given the association between endothelial dysfunction and CAD progression, **Chapter 5** explored relationships between FMD and arterial responses to exercise with the protein content of eNOS, NAD(P)Hox subunit 2, NFκB, ET-1, nitrotyrosine, the senescence markers (p53, p21, p16) and eNOS Ser^{1177}

phosphorylation in endothelial cells (EC)s obtained from the radial arteries of CAD patients. FMD was positively associated with eNOS Ser^{1177} phosphorylation, and protein content of p21 and p16, whereas no associations were found between FMD and markers of oxidative stress, vasoconstriction or inflammation. HE-induced dilation was not associated with any of the EC proteins, or FMD. A number of associations were observed between the expression of atherogenic risk-modulating proteins, providing novel insight into the molecular mechanisms related to vascular function in CAD. Lastly, **Chapter 7** provided novel molecular insights into how elevated shear stress induced by exercise benefits endothelial function in humans. In particular, it has been shown that a ~5-fold increase in shear stress induced by 30 minutes of HE resulted in upregulation of eNOS Ser^{1177} phosphorylation, whereas no effect was reported in PECAM-1 Tyr^{713} activation in ECs obtained from radial artery of young healthy well-trained males. As such, this study suggests that PECAM-1 activation is not involved in the vascular response to prolonged elevations in shear stress.

In conclusion, this thesis provided important information regarding the impact of catheterization on arterial function, showing preserved exercise-induced dilation in CAD patients following catheterization damage. This suggests early onset of exercise-based rehabilitation after catheterization procedures is safe, although this should be confirmed in other cohorts and in a larger sample. In addition, this thesis demonstrated novel insights into the molecular mechanisms related to arterial function in CAD, suggesting that progressive endothelial dysfunction in CAD may be more dependent on NO production than NO scavenging. Finally, this thesis provided novel data regarding the mechanism by which ECs sense elevated shear stress, suggesting no involvement of PECAM-1 in exercise-induced NO production.

AUTHOR DECLARATION

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

I declare that the work in this thesis was carried out in accordance with the regulations of Liverpool John Moores University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Liverpool John Moores University and the School of Sport and Exercise Sciences. This thesis has not been presented to any other University for examination either in the United Kingdom or overseas.

Signed: 

Date: 22/12/2019

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ABSTRACTS & CONFERENCE COMMUNICATIONS

During completion of this PhD at Liverpool John Moores University, data from this thesis resulted in the following abstracts and conference communications:

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Tryfonos A, Rodighiero R, Cocks M, Mills J, Green D.J, and Dawson E.A. Arterial *in vivo* and *in vitro* responses following catheterization-induced damage – Preliminary Results. Cardiovascular Symposium, Liverpool, UK 2018.

Tryfonos A, Rodighiero R, Cocks M, Mills J, Green D.J, and Dawson E.A. Determine the arterial *in vivo* and *in vitro* responses to catheterization-induced damage in patients and explore the effects of acute exercise to counteract these effects. Northern Vascular Biology Forum, Liverpool, UK 2017.

PEER-REVIEWED PUBLICATIONS

Tryfonos A, Green DJ, and Dawson EA. Effects of Catheterization on Artery Function and Health: When Should Patients Start Exercising Following Their Coronary Intervention? *Sports Med* 49: 397-416, 2019.

Tryfonos A, Cocks M, Mills J, Green DJ, and Dawson EA. Exercise-induced vasodilation is not impaired following radial artery catheterization in coronary artery disease patients. *Journal of applied physiology (Bethesda, Md : 1985)* 128: 422-428, 2020.

CHAPTER 1 - General introduction, Aims & Objectives

Cardiovascular disease (CVD) is the number one cause of morbidity and mortality worldwide [1]. Atherosclerosis is an inflammatory process which underlies most CVD conditions, resulting in thickening of the artery wall, plaque development and, sometimes, plaque rupture [2]. The endothelium, the inner monolayer of blood vessels, has emerged as a key regulator of vascular tone and homeostasis [2-4], whereas a dysfunctional endothelium has been associated with the development of atherosclerosis. Indeed, endothelial dysfunction appears to facilitate the transmigration and accumulation of white blood cells and vascular smooth muscle cells (VSMC) into the intima [2, 5, 6], resulting in plaque formation and stenosis.

Endothelial dysfunction is often characterised by a reduction in nitric oxide (NO) bioavailability. Endothelial nitric oxide synthase (eNOS) is the key enzyme for NO production [7]. However, reduced NO-bioavailability arises from not only impaired NO-synthesis, but also reflects NO-degradation by superoxide anions and related reactive oxygen species (ROS). Elevated oxidative status may also enhance the production of other vasoconstrictors or pro-inflammatory and senescence markers, contributing to further endothelial dysfunction and atherosclerosis progression [8]. Endothelial dysfunction appears to be clinically significant as a 1% decrease in endothelium-dependent dilation, assessed by brachial artery flow-mediated dilation (FMD), an indirect bioassay of endothelial dys/function, is associated with an 8-22% increase in risk of future CVD events [9, 10]. As such, there is increasing interest in studies assessing the role of the endothelium in CVD, with the ultimate aim to develop strategies to promote cardiovascular health by improving endothelial function.

There are many (patho)physiological mechanisms that lead to the gradual, long-term loss of endothelial integrity such as ageing [8] and/or elevated inflammation and oxidative status present during other conditions such as diabetes [11] or hypercholesterolemia [12]. However, vascular dysfunction may also occur acutely following arterial injury due to the mechanical removal or damage of the endothelial layer and may have several similar underlying pathways or lead to similar future risks for arterial function and health. For instance, catheterization procedures such as percutaneous transluminal coronary angiography (PTCA) and/or percutaneous coronary intervention (PCI; angioplasty), which are commonly used to diagnose and/or treat obstructed arteries, are associated with acute arterial injury, which is accompanied by structural and functional alternations such as neo-intima formation, thrombosis and endothelial dysfunction in treated coronaries (see review [13]). In fact, previous research suggests in-stent restenosis and stent thrombosis could present in 10% and 2% of the coronary target-vessel, respectively, which may result in a requirement for further revascularization procedures or myocardial infraction or cardiac death within 5 years [14]. A catheter is likely to mechanically disrupt or damage endothelial cells (EC)s. This endothelial dysfunction or damage can acutely convert dilator responses to increased flow and shear stress into constriction and possibly even spasm [15, 16]. Chronically, an injured or denudated endothelium permits the development of atherosclerosis, as it no longer provides an anti-thrombotic surface, and VSMC are exposed to the circulating blood, resulting in platelet binding and activation of the clotting cascade, possibly leading to thrombosis [17-19]. In addition, loss of endothelial function and cytokine release by platelets and macrophages further stimulates migration and proliferation of VSMC to the intima, contributing to the formation of a neo-intima layer and restenosis [20]. Given that coronary artery disease

(CAD) is a leading cause of death worldwide [21], and that PCI is the most common treatment for CAD [22] and one of most widely performed medical procedures in Western world [23], it is important to evaluate prevalent complications resulting from the catheterization-induced arterial injury such as restenosis and thrombosis, and to explore ways to minimize or achieve earlier recovery from such endothelial damage.

Exercise training has a profound impact on endothelial function in healthy individuals [24, 25] and CAD patients [26-37]. With respect to CAD in particular, exercise training has been associated with lower 5-year all-cause mortality in post-PCI patients [38], and is recommended as a key treatment for CAD [22]. A prominent mechanism through which exercise training leads to vascular adaptation is increase in shear stress. Shear stress is the tangential force of the flowing blood on the endothelial surface of blood vessels, and it is known that high shear stress promotes EC survival and enhances vasodilation. Conversely, low shear stress or turbulent flow results in apoptosis of ECs, along with VSMC proliferation and platelet aggregation [39]. To illustrate the importance of shear stress to vascular adaptation, Tinken *et al.* [40] clamped shear stress during exercise through cuff inflation, and found that the uncuffed (shear stressed) limbs exhibited greater improvement in endothelial function when compared with the cuffed limb at 2, 4 and 6 weeks of handgrip exercise training. This study was followed by other similar experiments in which increases in shear stress as a result of exercise, or even passive heating, were shown to modify arterial function and structure by virtue of their impacts on shear stress [41]. This reinforced the suggestion that shear stress is the key stimuli during exercise to improve endothelial function (see recent review [25]).

Whilst exercise training is generally recommended for CAD patients [22], catheterization-induced arterial damage in coronaries may transiently elevate the risk of cardiac events during exercise. Indeed, previous animal studies have demonstrated that catheterization results in ‘paradoxical’ vasoconstriction of damaged epicardial arteries in response to treadmill exercise [15]. If such constrictive responses are seen in human’s treated coronaries, there may be a basis to recommend a delay in the onset of cardiac rehabilitation, post-procedure in order to avoid triggering exercise-induced cardiac events. Therefore, it is important to evaluate the time-course of recovery from the “insult” of catheterization in treated coronary arteries, and whether exercise during this period presents a risk.

To evaluate the recovery of arterial function following catheterization, most studies have focused on the assessment of endothelium-dependent dilation, using invasive intravascular infusion of vasodilators within coronary arteries [42, 43]. However, given the inherent risk and invasive nature of evaluating coronary artery function following catheterization, less invasive procedures in the periphery have been suggested. In particular, assessment of radial artery function may represent a useful surrogate of coronary artery function, as both arteries are comparable in histopathology and size [44]. Indeed, a non-invasive technique of measuring endothelium-dependent dilation, called flow-mediated dilation (FMD) has previously been used to assess the recovery of vascular function following transradial catheterization [45-49]. Although invasive assessments of the coronary arteries and assessment of vascular function within the radial artery may provide useful information about arterial recovery, and therefore the safe onset of exercise rehabilitation in patients post-catheterization, to our knowledge there is currently no direct data on the response of human arteries to exercise stimuli

following catheterization-induced endothelial damage. Given the complex mechanisms by which exercise regulates blood flow [50], the vascular response of damaged arteries at rest may be different from the arterial response to exercise. Therefore, the first aim of this PhD thesis, presented in **Chapter 4**, was to examine conduit arterial responses to acute exercise pre- and post-catheterization in CAD patients. We hypothesized that vascular function, assessed via radial artery FMD, and response to handgrip exercise would be impaired 1-week following PTCA and/or PCI. If radial artery (surrogate to the coronary artery) response to exercise is impaired in the catheterized arm 1-week post procedure, it may suggest exercise rehabilitation following the procedure should be delayed until complete endothelial recovery has occurred to minimize the risk of exercise-induced cardiac events.

Subsequently, in the **Chapter 5**, the primary aim was to investigate whether the arterial response to FMD and handgrip exercise were associated with the expression and/or phosphorylation of endothelial proteins related to NO production, oxidative stress, vasoconstriction, inflammation and senescence pathways in ECs collected from the radial arteries of CAD patients. We hypothesised that FMD and exercise response would be positively associated with eNOS and its phosphorylation at Ser^{1177}, whereas it would be negatively correlated with atherogenic-modulating proteins related to oxidative stress, inflammation, vasoconstriction or senescence. A number of secondary aims and hypotheses are presented in Chapter 5.

In **Chapter 6**, we intended to examine the effects of endothelial damage-induced by mechanical injury (catheterization) on FMD in young trained individuals. To date all the data demonstrating depressed FMD following PTCA and/or PCI is reported in CAD

patients [46, 49]. As such, we wanted to examine whether such effects occurred in individuals with a fully intact functional endothelium or may be exacerbated in CAD patients due to *priori* endothelial dysfunction. Our hypothesis was that catheterization would result in either preserved or impaired endothelium-dependent dilation in response to shear stress in these healthy men. If shear-induced dilation is preserved in arteries with a fully functional endothelial layer, there may be an argument to apply pre-rehabilitation (exercise training) in patients prior to coronary interventions to reduced endothelial dysfunction related to arterial injury. In contrast, if vasodilation is impaired in apparently healthy arteries, similar to CAD, this may suggest that *priori* endothelial function does not matter in regards to catheterization-induced damage.

Lastly, in the **Chapter 7**, we aimed to explore the molecular mechanisms behind NO production in response to exercise in healthy young active males. In particular, we aimed to evaluate the role of platelet and endothelial cell adhesion molecule 1 (PECAM-1) in sensing shear stress and the subsequent downstream activation of eNOS. We hypothesized that PECAM-1 would be phosphorylated following 30 minutes of handgrip exercise in young trained males, and this would be associated with eNOS Ser^{1177} phosphorylation.

CHAPTER 2 - Literature Review

Part of this Chapter has been published:

Tryfonos, A., D.J. Green, and E.A. Dawson, *Effects of Catheterization on Artery Function and Health: When Should Patients Start Exercising Following Their Coronary Intervention?* Sports Med, 2019.

2.0. Cardiovascular diseases

Cardiovascular disease (CVD) is a general term referring to any class of disorder that results in poor function of the heart and/or blood vessels. Coronary artery disease (CAD), acute coronary syndrome (ACS), ischaemic stroke, heart failure, cardiomyopathy and peripheral artery disease are the main forms of CVD [51]. CVD is the number one cause for morbidity and mortality worldwide; an estimated 17.3 million people died from CVD in 2008, representing 31% of global deaths [29]. Deaths from CVD are predicted to increase to 23.6 million per year by 2030 [29].

2.1. Pathophysiology of atherosclerosis and the role of endothelium

Although the exact mechanisms that lead to CVD vary, depending on the disease in question, the majority of CVD deaths are linked to atherosclerosis development. Atherosclerosis (also known as arteriosclerotic vascular disease or ASVD), is an inflammatory process resulting in the thickening of arteries, plaque development and sometimes rupture, due to intramural invasion and accumulation of white blood cells, and proliferation of vascular smooth muscle cells (VSMC) in the sub-intima [2, 5, 6]. In the initial stages of atherosclerosis, abnormal function of the endothelium; the inner monolayer of blood vessels, can be identified [52, 53]. An intact endothelium is optimally placed to respond to physical and chemical signals. The endothelium produces a wide range of factors that regulate platelet adhesion and aggregation, vessel wall inflammation, growth factor production and VSMC proliferation [19]. A healthy endothelium is also responsible for regulating vascular tone by producing either vasodilator substances (e.g. nitric oxide (NO), prostacyclin (PGI_2)) or vasoconstrictor factors (e.g. thromboxane (TXA_2), endothelin-1 (ET-1)) [54]. The balanced production of these factors leads to a properly functioning atheroprotective

environment, whereas a damaged or dysfunctional endothelium may contribute further to the development of neo-intima formation and/or thrombus [54].

Endothelial dysfunction is involved in further atheromatous plaque formation by multiple mechanisms, including the up-regulation of adhesion molecules and platelet activation, increased cell permeability, enhanced low-density lipoprotein (LDL) oxidation, and VSMC proliferation and migration to intima [4, 55, 56]. Briefly, endothelial dysfunction is characterised by reduced NO production, which promotes increased macrophages and platelet adhesion to the vessel wall. A dysfunctional endothelium also has increased cell permeability which allows macrophages and LDL to enter the vessel wall. Macrophages produce reactive oxidative species (ROS) which affect the vessel wall contributing to further endothelial dysfunction, as ROS interacts with NO to form peroxide (ONOO-), leading to a further reduction of NO bioavailability. In addition, ROS enhances LDL oxidation, which furthers increases macrophage adhesion in the vascular wall. Macrophages react with oxidised-LDL and form foam cells, initiating atherosclerotic plaque formation [57]. In addition to macrophages, other types of cells such as ECs and VSMCs can became foam cells [57]. Endothelial dysfunction will contribute to further foam cell accumulation, leading to stenosis. In addition, since NO regulates VSMC proliferation, reduced NO bioavailability due to dysfunctional in ECs, increases the neo-intima layer [55]. There is a complex interaction between these processes which result in plaque formation with variable clinical outcomes [58]. With respect of CAD, most plaques remain asymptomatic, however some of them will eventually become obstructive, resulting in unstable angina pectoris, whilst others may rupture eliciting acute thrombosis leading to an ACS [58].

Taking into consideration the association between endothelial dysfunction and atherosclerosis [4, 55], there is a need to establish methods of measuring endothelial function and examine whether such measurements can be used to predict the risk of atherosclerotic disease. Indeed, endothelium-dependent dilation, assessed either invasively (coronary artery response to vasodilator infusion – e.g. acetylcholine (ACh)) [59], or non-invasively (flow-mediated dilation (FMD)) [60], is positively associated with the number of CVD risk factors in apparently healthy individuals. In addition, epicardial and microvascular coronary endothelial dysfunction independently predict acute cardiovascular events in patients with and without CAD [61]. A 1% decrease in peripheral endothelial function (FMD) has been associated with an 8-22% increase in the risk of future CVD events [9], and risk prediction appears to be stronger in diseased than healthy individuals [10]. Interestingly, endothelial dysfunction and the consequent risk of CVD events may be reversed by treating CVD risk factors such as diabetes, hypertension, hypercholesterolemia and tobacco smoking [4]. To conclude, atherosclerotic diseases have become a dominant and compelling public health problem, and endothelial dysfunction appears to be a critical factor in the development of such diseases, therefore it is critically important to work towards the improvement of endothelial function in order to manage CVD [58].

2.2. The molecular mechanisms of endothelial (dys)-function

There are numerous molecular mechanisms that can lead to endothelial dysfunction. These can include reduced NO production, increased levels of inflammation, oxidative stress, senescence or vasoconstrictive molecules such as endothelin-1 or thromboxane A_2 (TXA_2) [8, 55, 62]. Indeed, one of the most important functions of

endothelial cells (EC)s is the production of NO, which leads to VSMC relaxation (vasodilation), whereas inhibition of NO results in reduced vasodilation or vasoconstriction [54]. In addition to vascular tone regulation, NO is known to control vascular wall inflammation, inhibit platelet aggregation and modulate VSMC proliferation, which all inhibit formation of thrombus and plaques [63]. Indeed, endothelial dysfunction and the development of CVD have been associated with reduced NO bioavailability [55]. Reduced NO bioavailability may be a result of lower NO production but also increased NO scavenging[64]. A number of mechanisms contribute to decreased NO production and/or NO scavenging, including reactive oxidative species (ROS), inflammation or cellular senescence. More importantly, such mechanisms are interlinked, indicating a complex vicious cycle of endothelial dysfunction. Below, we present some of the most important molecular mechanisms participating in the development of endothelial dysfunction which are related to the following Chapters.

2.2.1. Endothelial Nitric Oxide Synthase (eNOS)

Endothelial nitric oxide synthase (eNOS), is the rate limiting enzyme responsible for the NO production from the L-arginine in ECs. The combination of eNOS protein content, and its activation state, determine NO production. Although a number of molecular events regulate eNOS, its phosphorylation on multiple sites has been proposed as a key mechanism for eNOS activation. eNOS can be regulated by multiple phosphorylation sites at tyrosine, serine, and threonine residues, which may be activated by different stimuli and contribute to different functions. For instance, eNOS phosphorylation at Ser^{1177} (peNOS Ser^{1177}) appeared to upregulate eNOS, while phosphorylation at Thr^{497} may inhibit eNOS activity, resulting in decreased NO

production [65]. eNOS Ser^{1177} phosphorylation has emerged as the most researched site as it could be activated by a number of important signals, including elevated shear stress (frictional force of the flowing blood) [66]. Indeed, using animals transduced with a non phosphorylatable eNOS gene it was shown that acetylcholine-mediated vasodilatation was significantly reduced compared to animals transduced with an eNOS phosphorylatable at Ser^{1177} [67].

Reduced NO production by eNOS may impact endothelial function and thus contribute to CVD development. Studies in eNOS knockout mice suggest these animals experience impaired vasodilation [68] and enhanced vasoconstriction [69] and are more likely to develop a CVD phenotype [7], including hypertension [68]. However, other studies suggest that despite the impaired ACh-induced vasodilation observed in hypercholesterolemic [70] and aged mice [71], the arterial expression of eNOS was not affected, raising questions about the role of eNOS content in the development of endothelial dysfunction.

There is limited data on the expression of EC proteins, including eNOS, in conduit arteries of humans. This is due to limited availability of endothelial tissue for use when investigating the cellular mechanisms of endothelial dysfunction in patients with CVD. To address this, early work used arteries harvested during surgical procedures. Such data has shown that eNOS expression was lower in atherosclerotic arteries compared to angiographically normal arteries (carotid or mammary arteries) obtained during carotid atherectomy or CABG [72]. In addition, coronary arteries obtained from heart transplant patients had lower eNOS gene expression in patients with advanced

disease (dense fibrosis or macrophage accumulation) than those with early atherosclerosis (only VSMC layer) [73]. Remarkably, Rossi *et al.* [74], showed that plaques from patients with acute coronary syndromes (ACS) had higher eNOS gene expression, when compared to plaques from stable angina patients. The authors suggested that higher eNOS expression in ACS patients may represent a protective mechanism against the sudden expansion of an atheromatous lesion.

Recent work has developed a method to sample ECs from conduit arteries through catherization and J-wires. Using this technique Colombo *et al.* [75] showed that eNOS protein content was not different between patients with chronic heart failure and healthy controls, in ECs obtained from an antecubital vein. Similarly, ECs obtained from the brachial artery demonstrated no significant difference in eNOS content in humans with CVD risk factors, such as ageing [76] and obesity [77], when compared to young or lean subjects, respectively. As such, the authors suggest that altered eNOS expression does not contribute to endothelial dysfunction in ageing or obesity. To conclude, animal knockout of eNOS results in endothelial dysfunction and the development of CVD, but data from humans suggests the role of eNOS content in CVD may be dependent on the severity of cardiovascular disease, as eNOS is unchanged in obesity and ageing (risk factors for future cardiovascular disease), but is reduced in patients with diagnosed CVD. Moreover, the variable outcomes reinforce the importance of conducting human studies when feasible, as studies in animals do not always predict what is seen in humans.

As well as altered eNOS content reduced eNOS phosphorylation may be key to impaired NO production in subjects with endothelial dysfunction and/or disease.

Indeed, data from EC culture models illustrated reduced eNOS Ser^{1177} phosphorylation in aged human umbilical vein and arterial ECs [78, 79], and arterial ECs under hyperglycemia [80]. In addition, there was a reduction in aortic eNOS Ser^{1177} phosphorylation in older mice compared to young controls [71]. However, eNOS Ser^{1177} phosphorylation has been shown to be increased in human ageing and obesity compared to young or lean individuals, respectively [76, 77]. It has been hypothesized that this increase in eNOS activation in humans might represent a compensatory mechanism to maintain NO bioavailability when NO scavenging through oxidative stress is elevated [81]. Indeed, hydrogen peroxide (H_2O_2) has been shown to phosphorylate eNOS at Ser^{1177} [82]. To conclude, there is conflicting data regarding eNOS content and its activation at Ser^{1177} in CVD, with animals and cell culture demonstrating reduced eNOS content and eNOS Ser^{1177} phosphorylation, whereas unchanged eNOS content and elevated eNOS Ser^{1177} phosphorylation has been reported in humans with CVD risk factors. This might suggest that rather than eNOS content, its activation in response to stimuli may play a role in maintaining NO-production in humans with CVD.

2.2.2. Oxidative stress

Reduced NO bioavailability may arise not only from impaired NO synthesis, but also reflects NO scavenging by superoxide anions and related reactive oxygen species (ROS). As such elevated oxidative stress has been implicated in the development of endothelial dysfunction and atherosclerosis [83]. Shishenbor *et al.* [84] demonstrated that systemic circulating nitrotyrosine is elevated in CAD patients, and patients in the upper quartile for circulating nitrotyrosine had higher odds of CAD compared with those in the lowest quartile. Importantly, Thomson *et al.* [85], reported a strong

association between circulating nitrotyrosine levels and angiographic evidence of significant CAD. NT is the stable end product formed during the oxidation of NO [85], when superoxide anions (O_2^-) react with NO and form peroxynitrite (ONOO-), an unstable product that quickly reacts with tyrosine to form nitrotyrosine. Thus, NT is traditionally known as the footprint of oxidative stress in the vasculature [86]. In addition, Beckmann *et al.* [86] demonstrated pronounced abundance of nitrotyrosine in human atherosclerotic plaques. Finally, abundance of nitrotyrosine was found to be higher in ECs obtained from subjects with CVD risk factors, such as obesity [77] and ageing [87], and was inversely associated with endothelial function (FMD) [88, 89]. Although the above associations are between markers of oxidative stress and endothelial dysfunction, this does not necessarily imply cause and effect. However, this data may suggest the involvement of oxidative stress in the development of endothelial dysfunction, and ultimately atherosclerosis and CVD.

Although a variety of enzymatic and non-enzymatic sources of ROS have been detected in blood vessels [90], nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)Hox) has been reported as the primary source of ROS, particularly superoxide production in the vasculature [91]. Nicotinamide adenine dinucleotide phosphate oxidase (NAD(P)Hox), are a family of multi-subunit enzyme complexes which catalyse the reduction of molecular oxygen using NAD(P)Hox as an electron donor, generating superoxide [92]. In non-phagocytic cells such as ECs, NAD(P)Hox is expressed at low levels, adequate for the controlled cellular metabolism, and thus, ROS is restricted. However, in pathological conditions, such as hypertension, diabetes and hyperlipidaemia, the activity of NAD(P)Hox is considerably increased, resulting in overexpression of ROS [91]. The NAD(P)Hox complex is made of a core membrane

bound catalytic subunit (endothelial cells express 4 NOX isoforms NOX1, 2, 4 and 5) and up to five regulatory subunits. These regulatory subunits have important roles in: the maturation and expression of the NOX subunits (p22phox, DUOX activator 1 (DUOXA1) and DUOXA2); in enzyme activation (p67phox and NOX activator 1 (NOXA1)); and in spatial organization of the various components of the enzyme complex (p47phox, NOX organizer 1 (NOXO1) and p40phox). In regards to the catalytic subunit of NAD(P)Hox, NOX2 was the first NOX isoform identified in ECs and is likely to be the most important subunit in the production of superoxide in the vascular wall [91].

Judkins *et al.* [93], demonstrated higher expression of NOX2 but not NOX4, in aorta of aged, atherosclerotic-prone mice (ApoE $^{-/-}$), when compared to age-matched controls. More importantly this study showed that NOX2/ApoE double knockout mice (NOX2 $^{-/y}$/ApoE $^{-/-}$), had ~50% lower aortic atherosclerotic lesion formation and ~25% lower superoxide production, compared to single ApoE knockout mice (ApoE $^{-/-}$), illustrating the role of the NOX2 subunit in ROS production and subsequent development of atherosclerosis [93]. In addition, NOX2 gene expression in human coronary arteries obtained during heart transplant was associated with atherosclerosis severity (as defined by American Heart Association classification guidelines for atherosclerotic lesions) [94]. Systemic NOX2 activation, as expressed by soluble NOX2 derived peptide (sNOX2-dp), was higher in patients with peripheral artery disease, compared to matched controls, and NOX2 activation was inversely correlated with endothelial function (FMD) [95]. Finally, Loffredo *et al.* (2013), recruited patients with hereditary deficiency of NOX2 (known as the chronic granulomatous disease) and aged matched healthy and obese controls, in order to address the relationship

between oxidative stress and endothelial function. They reported that these patients had lower systemic markers of oxidative stress (urinary isoprostanes) and higher serum markers of NO bioavailability (serum nitrite/nitrate) and endothelial function (FMD) than controls [95]. Collectively, these studies indicate that increased NAD(P)Hox, and specifically endothelial expression of its NOX2 subunit, leads to increased ROS production which has been associated with impaired endothelial function and progression of atherosclerosis.

Lastly, although NAD(P)Hox has been shown to be the primary mechanism of elevated oxidative stress in the vasculature, other mechanisms including xanthine oxidase, inactivation of antioxidant systems and uncoupling of eNOS via tetrahydrobiopterin (BH_4) oxidation, have also been implicated in elevated $O_2^{\cdot -}$ production and NO scavenging [96].

2.2.3. Inflammation. The role of NFκB

In addition to oxidative stress, inflammation in the vascular wall plays an important role in atherosclerosis development [97, 98]. Leukocytes and proinflammatory cytokines are actively involved in the formation of foam cells in the intima, which are the foundation of atheromatous plaques [97]. An intact endothelium does not bind leukocytes, however, from the initial stages of endothelial dysfunction, ECs begin to express on their surface adhesion molecules, resulting in binding of leukocytes [99]. Leukocytes can then enter the vascular wall and form foam cells which will continue to secrete inflammatory cytokines, ROS and other atherogenic mediators, contributing to plaque development [97]. Importantly, ECs not only respond to cytokine signals, but

they also produce pro-inflammatory markers [99], indicating the early role of inflammation in endothelial dysfunction and atherosclerosis.

Inflammation, at both focal and systemic levels, plays a key role in destabilization and rupture of atherosclerotic plaques, leading to acute cardiovascular events [99]. Kaski *et al.* [100] demonstrated higher incidences of acute coronary events in patients with "complex" coronary stenosis morphology, compared to patients with smooth lesions. The complexity of coronary plaques has been associated with higher levels of inflammatory markers [101]. Indeed, higher expression of pro-inflammatory markers such as interleukin 6 (IL-6), tumour necrosis factor-1 alpha (TNF-1α), c-reactive protein (CRP) and nuclear factor κB (NFκB) etc., have been used to predict cardiac events in CAD patients [102-104] and in apparently healthy men [105] and women [106]. Of importance, inflammatory markers have also been negatively associated with endothelial function as assessed by brachial artery FMD [107], which suggests that endothelial dysfunction may be a link between inflammation and progression of atherosclerosis.

Although several inflammatory markers have been identified in the development of atherosclerosis, of particular interest for this PhD thesis is NFκB, due to its interactions with other oxidative and pro-inflammatory pathways. NFκB is an important redox-sensitive transcription factor which is produced by ECs and plays a key role in endothelial dysfunction. It has been proposed that NFκB promotes endothelial dysfunction, and subsequently atherosclerosis, thought its role in regulating gene expression of factors that control inflammation, cell adhesion and oxidative stress

[108-110]. Earlier studies demonstrated the presence of NFκB in human atherosclerotic regions [111, 112], suggesting its role in atheromatous formation. Indeed, endothelial specific inhibition of NFκB activation in atherosclerotic-prone mice (ApoE $^{-/-}$), resulted in reduced plaque formation, lower extent of macrophage recruitment in plaques and reduced inflammation in aorta [113].

With respect to humans, elevated NFκB expression has been observed in peripheral blood mononuclear cells (PBMC) in CAD patients [114] and in patients with familial hypercholesterolaemia [115], when compared to matched controls. To our knowledge, there is no evidence showing NFκB levels in ECs of CAD patients and whether this is altered due to the disease progression. The most relevant data we currently have are studies including subjects with CVD risk factors, indicating higher NFκB expression in ECs of obese [77] and older people [88], compared to lean and young individuals respectively. Although somewhat speculative, these results suggest the potential role of NFκB in endothelial dysfunction. In this context, Donato *et al.* [88] shown that NFκB expression in ECs was positively associated with nitrotyrosine level and was inversely associated with FMD, suggesting a vicious cycle between inflammation and oxidative stress in endothelial dysfunction. Indeed, previous work demonstrates the potential for NAD(P)Hox dependent induction of NFκB [116]. To further support the above, NFκB inhibition (Salsalate administration) resulted in reduced NOX2 expression and improved endothelium-dependent dilation, in obese subjects [117]. Collectively, these findings suggested that NFκB is an important transcription factor which link the pro-oxidative/inflammatory endothelial phenotype with endothelial dysfunction and atherosclerotic diseases.

To conclude, inflammation has a crucial role in not only the early stages of atherosclerosis development but also in the rapid progression and the complexity of atheromatic plaques. Endothelial dysfunction has been suggested as one of the links between inflammation and atherosclerosis. Evidences suggest that the level of pro-inflammatory markers may be useful in the prediction of cardiac events in CAD patients. However, further large-scale studies are required to identify which marker is the most suitable and the threshold among low- and high-risk cases.

2.2.4. Endothelin-1

ECs not only produce vasodilators such as NO, but they also secret vasoconstrictors. Endothelin-1 (ET-1) is the most important vasoconstrictive agent produced by ECs [118]. Interestingly, unbalanced endothelial production of NO and ET-1, has been proposed as an alternative definition for endothelial dysfunction [62]. An intact endothelium produces limited amounts of ET-1, while excessive ET-1 expression has been reported in endothelial dysfunction [119]. ET-1 acts via two receptors, $ET\text{-}1_A$ and $ET\text{-}1_B$ receptors. The $ET\text{-}1_A$ receptor is located in VSMC where they are responsible for potent vasoconstriction. $ET\text{-}1_B$ receptors are primarily found in ECs and its stimulation results in NO production. Interestingly, $ET\text{-}1_B$ receptors are also expressed in VSMC where their activation results in vasoconstriction. Therefore, ET-1 vasoconstrictor function is determined by receptor localization and the balance between $ET\text{-}1_A$ and $ET\text{-}1_B$ receptors [119]. Alongside ET-1's direct function as a vasoconstrictor, ET-1 may affect NO production by a number of mechanisms, including oxidative stress and inflammation [120, 121].

Circulating ET-1 has been shown to be significantly elevated in symptomatic CAD patients, when compared to healthy controls, and the ET-1 level was positively correlated with the number of atherosclerotic plaques [122], indicating its role in atherosclerosis progression. In addition, circulating levels of ET-1 are higher in stable CAD patients who experienced a cardiac event during 24 months follow-up, compared to non-event patients [123]. Higher ET-1 expression was also observed in ECs of old people compared to young individuals, supporting the association between ET-1 and CVD risk factors [76]. Jankowich *et al.* [124] summarizing the existing literature regarding the role of ET-1 in cardiovascular risk prediction. Similarly, Rich and McLaughlin payed attention to the potential role of blockage of ET-1 receptors, and concluded that although the most of data examined the efficiency of receptor $ET\text{-}1_A$ blockage, it remained unclear whether selective $ET\text{-}1_A$ or nonselective blockade is superior in improving endothelial function and benefit CVD.

In particular, 6-month administration of an $ET\text{-}1_A$ receptor antagonist in patients with multiple CVD risk factors resulted in improved endothelium-dependent dilation of coronaries in response to ACh [125], suggesting the role of ET-1 in endothelial dysfunction. Conversely, intravascular infusion of ET-1 in young healthy subjects resulted in impaired endothelium-dependent dilation (venous occlusion plethysmography), and increased circulating IL-6 [120]. Importantly, endothelium-dependent dilation was preserved with vitamin C administration prior to ET-1 infusion [120], implying the potential involvement of oxidative stress and inflammatory pathways in ET-1-mediated endothelial dysfunction. Finally, Donato *et al.* [76] demonstrated an inverse correlation between ET-1 expression in ECs and endothelium-dependent dilation, assessed by FMD.

2.2.5. Cellular senescence

Increasing age is an independent risk factor for CVD and it has been associated with reduced endothelial function [88, 126]. Despite the traditional mechanisms involved in age-related endothelial dysfunction, other mechanisms such as cellular senescence appeared to be involved in endothelial dysfunction and thus CVD [127]. Cellular senescence is a stress-response resulting in irreversible growth arrest of a cell, and indeed, it has been emerged as a potential driver of several diseases, including atherosclerotic diseases [128].

Although the exact mechanisms by which cells become senescent are not fully understood, signals eliciting cellular stress such as DNA damage, oxidative stress, inflammation, ageing, and dysfunctional telomerases all appear to stimulate senescence [128-130]. Senescence growth arrest is mediated through two main pathways, p53/p21 and p16 [128, 131]. Indeed, Suzuki *et al.* [132] showed that elevated oxidative stress (H_2O_2 treatment) in HUVECs induced higher expression of p21, p53 and p16, demonstrating the relevance of such proteins in EC senescence. Notably, while the activation of either pathway results in deceleration of the cell cycle, these pathways are not completely equivalent as different stimuli, cell type or species may initiate distinct pathway and lead to various cell responses [129]. See the review of Campisi *et al.* [129] for more information about the role and stimulation of each pathway.

Senescent cells secrete pro-inflammatory and pro-oxidative factors, known as senescence-associated secretory phenotype (SASP). Accumulation of senescent cells in addition with the SASP of these cells, have been proposed as the potential

mediators of atherosclerosis development [133, 134]. To support the above, there was higher accumulation of senescent cells in atherosclerotic lesions of atherogenic-prone mice (low-density lipoprotein receptor-deficient mice (Ldlr $^{-/-}$)), fed a high fat diet, compared with those fed a low fat diet [135], indicating the contribution of cellular senescence in the atherosclerosis process. More importantly though, this study reported an elevated transcription of pro-inflammatory molecules such as TNF-1a in senescent cells [135], reinforcing the role of SASP in the development of atherosclerosis. In addition, IL-1β expression was higher in oxidative stress-induced senescent HUVECs compared to the control culture [132]. Finally, IL-1β was expressed in senescent cells located in human atherosclerotic lesions, which further supports the above [136]. NFκB is also known to initiate and maintain the SASP [133, 137], and it is known to be involved in atherosclerotic process (see above section 2.2.3).

Although there is evidence suggesting a contribution of senescence to atherosclerosis, there are limited studies providing direct data in regards to EC senescence and how this interferes with endothelial function. In particular, evidence is limited to ageing research, demonstrating higher expression of senescence markers (p53, p21 and p16) in ECs obtained from older subjects [126], and aortic tissues of aged mice (p16, p19) [138], when compared to young controls. Importantly, the expression of senescence-associated markers was inversely associated with endothelium-dependent dilation in both humans and mice [126, 138]. These findings further support the suggestion that cellular senescence may play a role in endothelial dysfunction and subsequent atherosclerosis development. Lastly, it is worth noting that although EC senescence appears to play a role CVD, largely due to the SASP, there is currently limited

information about endothelial senescence in patients with documented CVD, and whether this is different compared to age-matched controls. Therefore, further studies in humans are needed to provide direct evidence of the contribution of senescence in age-matched patients with and without CVD.

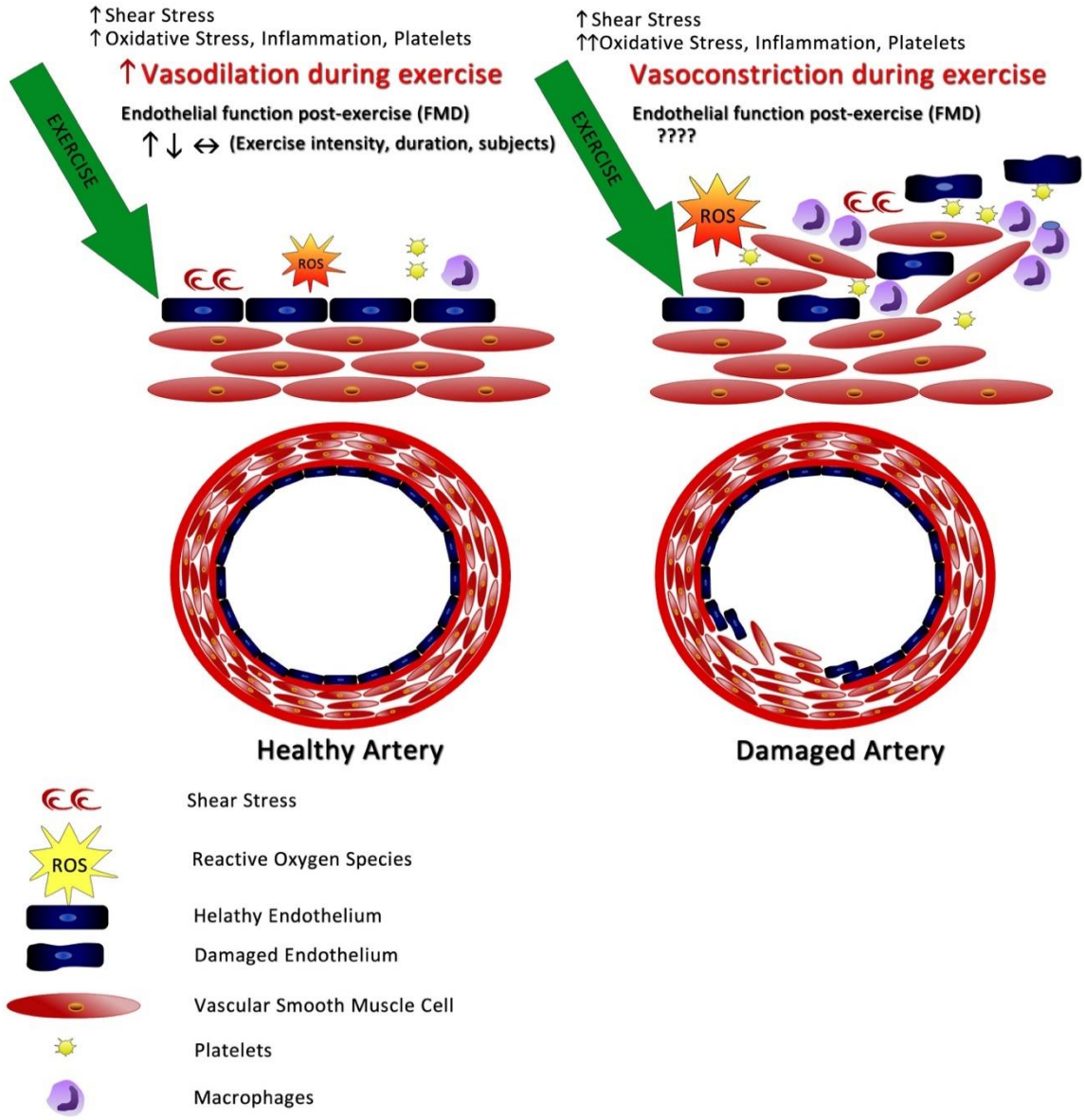
2.3. Coronary artery disease

The presence and extent of coronary artery plaque and associated stenosis is commonly determined using percutaneous transluminal coronary angiography (PTCA), a technique that utilizes contrast agent and X-ray based detection to visualize the vessel lumen [139]. Treatment for an obstructed artery can include angioplasty (PCI; percutaneous coronary intervention); where a catheter with balloon tip is inserted and advanced to the stenosed coronary artery. A balloon is inflated to restore the blood flow and a stent (expandable tube-shaped device), is commonly deployed inside the artery to keep it open. PCI is the most common treatment of CAD [22] and one of most widely performed medical procedures in Western world; with 97,376 PCI reported in the UK in 2015 [140], although it is worth noting that critical appraisal of the benefits of PCI, relative to conservative medical management or CABG, has only recently begun to emerge [141, 142]. In particular, PCI appeared to have similar results in exercise-induced ischemia compared to patients who undertaken placebo PCI, suggesting that perhaps medical treatment could equally manage stable CAD patients [142]. Earlier, Hambrecht *et al.* [143] shown that compared with PCI, a 12-month program of regular exercise resulted in superior event-free survival, improved exercise capacity and lower costs due to reduced hospitalizations and repeat revascularizations. In addition, randomized trial comparison between PCI and CABG have not demonstrated a conclusive choice for which produces superior outcomes

[144]. In general, CABG may have superior long-term outcomes in more complex anatomic CAD disease, whereas PCI is generally preferable for patients with more non-cardiac comorbidities at least short term [144]. Technological advances have improved both PCI and CABG procedures, therefore, high-quality randomised clinical trials could be conducted to examine the benefits and risks related in both treatments short term and long term.

2.3.1. Do coronary interventions cause arterial injury?

Percutaneous transradial coronary artery procedures (PTCA) and/or PCI are considered safe and effective [145, 146], however, vascular dysfunction and other complications such as re-stenosis and thrombosis are well recognized [17]. There is clear evidence that invasive procedures mechanically disrupt and can damage the vascular endothelium. Acutely, endothelial dysfunction or damage can convert dilator responses to increased flow and shear stress into constriction and possibly even spasm in denudated arteries. Chronically, an injured or denudated endothelium permits the development of atherosclerosis, as it no longer provides an anti-thrombotic surface, and VSMC are exposed to the circuiting blood, resulting in platelet binding and activation of the clotting cascade, possibly leading to thrombosis [17-19]. In addition, loss of endothelial function and cytokine release by platelets and macrophages further stimulate migration and proliferation of VSMC to the intima, contributing to the formation of neo-intima layer and restenosis in the treated (damaged) arterial section [20] (Figure 2.1).



<u>Figure 2.1.</u> Mechanisms of artery responses to exercise with and without endothelial denudation.

Exercise typically induced vasodilation (increase arterial diameter) in healthy arteries (left). Shear stress is elevated during exercise, which will result in increased dilation due to an increase in NO production [147]. Oxidative stress, inflammation and platelets (count/agreeability) may be increased during exercise, particularly following strenuous exercise. Endothelial function immediately post-exercise, assessed by flow mediated

dilation (FMD), is equivocal and has been shown to increase, decrease or not change. This variation may relate to exercise intensity, type, duration and the subject's fitness level [148]. The damaged artery, following catheterization (right) will result in paradoxical vasoconstriction [15]. Even though there is an increase in blood flow and shear stress during exercise, the absence of endothelium in the damaged artery can abolish the dilatory response of artery to an exercise [16]. Higher levels of oxidative stress, inflammation and platelets are typically presented in damaged arteries post-catheterization. Endothelial denudation will also result in vascular smooth muscle cell (VSMC) proliferation and migration into the intima, leading to neo-intima formation. This arterial stenosis appeared to be associated with the degree of endothelial damage, indicating higher neo-intima formation in arteries with larger degree of endothelial denudation [149]. There is no information yet in regards to endothelial function post-exercise in damaged arteries.

2.3.1.1. What are the effects of catheter insertion, balloon inflation and stent implantation on arterial structure and function?

Structural changes.

Whilst catheterization on its own (i.e. PTCA) may damage the coronary artery, the addition of balloon inflation and stent deployment may cause a greater degree of injury, resulting in neo-intima thickening [150]. Neo-intima thickening appears to depend on the degree of endothelial denudation [149], a small denuded area results in little intimal hyperplasia [151], while larger denuded areas lead to greater intima thickening [152]. Newer, 2nd generation bioresorbable drug-eluting stents (DES), with anti-inflammatory and anti-proliferative properties, have shown lower neo-intima

thickening [153], and superior clinical outcomes [154] including reduced mortality, morbidity [155], and revascularization rates [156]. However, the incidence of in-stent restenosis [157, 158] and late thrombosis [159, 160] is still an issue. Indeed, approximately 10% and 2% of the target-vessel will present in-stent restenosis and stent thrombosis respectively, which may result in further revascularization procedures, myocardial infraction or cardiac deaths within 5 years [14]. Recently, stents with seeding cells, endothelial progenitor cells (EPC) or ECs, have been studied utilizing *in vitro* and *in vivo* models, with promising outcomes regarding in-stent restenosis [161, 162]. Further research and clinical trials are needed in this area.

To evaluate the extent of arterial injury as well as the incidences of stenosis in coronaries following catheterization, a follow-up coronary angiography is usually required. Given the invasive nature and perhaps unnecessary risks revealed from the angiography, less invasive methods that measures the structural alternations in peripheral arteries has been suggested [46, 49, 163]. Specifically, the radial artery has been proposed as a useful surrogate of coronary arteries, as they are comparable in histopathology and size [44]. Indeed, the radial artery injury has been identified in approximately 1/5th of patients following transradial procedures, 1/3rd of which have significant dissection extending into the media layer [48]. Moreover, greater extent of injury in radial arteries has been associated with repeated catheterization [164]. The radial artery can therefore provide insight into acute and long-term structural changes, post-catheterization which may be applicable to coronary arteries. In addition to being a potential surrogate for the coronary arteries, the radial artery it has been proposed as the best second coronary artery bypass graft [165-168] and therefore understanding of the damage that prior catheterization may have on the artery is

useful. This may impact on choice of either catheterization site or whether it can be a viable donor graft for bypass surgery.

Intima media thickness (IMT), a non-invasive assessment of atherosclerosis, has been shown to increase in catheterized radial arteries at 1- [169], 3- [170] and 4-months [171] following radial procedures, potentially indicating a chronic impact on the structure of the artery wall. Interestingly, IMT has also been shown to increase after only 1 day [169], which likely reflects the acute impact on smooth muscle contraction, which itself can increase wall thickness measures [172]. Previous catheterizations may result in long-term structural remodeling, either due to an increased IMT or a decreased diameter [167, 169, 170, 173], which may have consequences for the long-term patency of the vessel. If the radial artery is removed and used as a graft for CABG, the stenosis-free graft patency is higher if the artery had not been previously catheterized for angiogram/PCI [167].

To summarize, there is evidence for an initial increase in artery diameter due to catheter insertion. Long term, further IMT thickening and decreases in diameter may evolve. In additional to structural changes, PCI may also affect arterial function. Structure and function interact [25], as reduced endothelial function post-injury has been associated with the degree of intimal thickening [174, 175].

Functional changes.

Numerous methods can be used to assess vascular function, either invasively or non-invasively, in both coronary and peripheral arteries. The most common method to evaluate coronary artery function to date has involved intracoronary infusion of endothelium-dependent and –independent dilators and quantitative angiography. More recently, non-invasive techniques (cardiac magnetic resonance imaging, positron emission tomography, and computed tomography) have been used to assess changes in coronary diameter.

Impaired endothelium-dependent and -independent dilation, and paradoxical constriction are major functional complications following PTCA and/or PCI in coronary arteries [15, 42, 150, 174, 176-179]. The grade of endothelium-dependent dysfunction may depend on the severity of denudation [179], whereas VSMC function appears not to be related with the extent of denudation [174]. Plain-balloon injury, bare-metal stents (BMS) and drug-eluting stents (DES) all result in acute endothelial and VSMC dysfunction [150, 180]. In addition to the directly stented section, proximal and distal segments may experience impaired vascular responses [43, 181, 182]. Greater dysfunction occurs distal than proximal to stented area [183-186], resulting in greater vasoconstriction [42] and distal coronary vasospasm [187], potentially increasing thrombotic risk. Moreover, endothelial dysfunction assessed in this distal section of the vessel has been associated with poor endothelial coverage in this area [185].

In general, direct measurement of coronary artery function can be difficult and is highly invasive. Over the past few decades, a non-invasive assessment, called flow-

mediated dilation (FMD), has emerged to assess endothelium-dependent function in humans, usually in the brachial artery. The change in artery diameter in response to sublingual-glyceryl-trinitrate spray (GTN) is also commonly used to evaluate endothelial-independent, but nitric oxide (NO)-mediated, VSMC function. Similarly, FMD and dilation to GTN have been used to examine the effects of catheterization in the peripheral denuded/damaged arteries following PTCA and/or PCI, in order to avoid follow-up coronary angiographies [46, 49, 163]. Although radial arteries are comparable in size and histopathology with coronaries [44], and therefore could be used as a useful surrogate, the extent of radial artery injury is limited to the catheter insertion while coronaries are likely to experience a greater degree of injury due to balloon inflation and/or stent placement in case of PCI. Therefore, the effects of catheterization on peripheral arteries could provide useful information of arterial injury, however they should be interpreted with caution. There is clinical relevance for determining the extent of injury to the radial artery during catheterization as the artery is sometimes used as a donor graft for CABG [165-168]. FMD has been shown to be acutely lower in catheterized radial arteries compared to the non-catheterized arteries [45-49]. In general, equivocal findings regarding radial artery function post-catheterization are reported, with a reduction noted at 4- [48], 9- [47] and 12-weeks [45] in some studies, whereas others suggest that vascular function returns to baseline at 3- [46, 48, 49] and 12-months [188]. In addition to dilatory function, the ability of the artery to constrict is also reduced 24h and 7-weeks post-catheterization [163]. However, VSMC function appears to recover earlier than endothelial function, with reduced function at 24 hours [45-48], and 1 week [48] but not 1 and 3 months [48] (Figure 2.2).

Whilst change in localized function or radial (and possibly coronary arteries) is likely due to direct damage, there may also be a systemic effect of catheterization. Lower brachial artery function following PCI, assessed in the non-catheterized vessel, was associated with late in-stent restenosis at 1-month [189], 6-month [175] and 12-month [190, 191]. In contrast, VSMC function was unrelated to the incidence of in-stent restenosis [189]. Such vascular responses were independent of stent type [191]. Given that preserved function is often reported in a non-catheterized control limb [48, 49], further work is needed to determine if there is a systemic effect of PCI and whether inflammatory or oxidative stress mechanisms are involved.

In general, evidence suggests that endothelial denudation following endovascular procedures impairs vasomotor function, while VSMC function is often relatively preserved. It remains unclear whether such interventions result in systemic vasomotor dysfunction, but some indicative evidence suggests that this may be the case. Endothelial dysfunction in coronary arteries, assessed by ACh infusion has been associated with 9-13% cardiac event risk and 21% revascularization requirement in 8-year follow-up [61]. Similarly, a 1% decrease in peripheral endothelial function (FMD) has been associated with 8-22% increase in risk of future CVD events [9], and risk prediction appears to be stronger in diseased than healthy individuals [10]. Although the above studies include CAD patients, it is unclear whether they had catheterization procedures. However, FMD of peripheral arteries post-PCI (non-catheterized brachial artery following femoral PCI) appeared to have a clinical significance as it has been associated with late restenosis or revascularization rates (coronary arteries) [189, 192, 193]. Similarly, patients with stenosis post-PCI had lower coronary blood flow [194],

which further supports the potential relevance of measuring peripheral artery function to mirror coronary endothelial function.

2.3.1.2. Mechanisms leading to neo-intima formation and vasomotor dysfunction post-PTCA and/or PCI

Increased oxidative stress and inflammation post PTCA/PCI has been associated with restenosis and vascular dysfunction [195-202]. Neo-intima formation (and VSMC proliferation) within the first week [151, 203] may be a consequence of the release or synthesis of growth factors by VSMC, including PDGF (platelet-derived growth factor; a potent VSMC mitogen), while the elevated VSMC proliferation in the following weeks [20, 151] may be explained by reduced NO production due to endothelial damage [204]. Loss of eNOS and reduced NO production post-injury initially occurs due to the actual damage/death of ECs during the procedure, and subsequently due to increased production of reactive oxygen species (ROS) [196, 197, 205-207], which appears to follow a similar time course to proliferation post-injury [208]. ROS production and inflammation increase immediately after vascular injury (following both PCI and PTCA [199]) and remain elevated for hours [197] or even days (1-15 days) [195, 201, 209, 210], returning close to baseline levels at 1-month [211]. Inflammation, oxidative stress and platelet adhesion are associated with endothelial dysfunction in CAD patients [212] and increased radial IMT in post-PCI patients [171]. In further support of this, some studies indicate that antioxidant treatments in both animals and patients are associated with reduced neo-intima formation [201, 209, 211, 213-216], greater luminal diameter, and lower risk of major adverse cardiac events [215]. Finally, lower antioxidant capacity in post-PCI patients is a predictor for cardiac event risk [217].

2.3.2. Recovery of function and structure following catheterization.

In animal models, impaired endothelium-dependent dilation of catheterized arteries is evident in the first days to 4 weeks [15, 16, 150, 174] and tends to recover by 4-8 weeks [177, 218]. In contrast, reduced VSMC function is apparent only the first week post-denudation [15, 150, 177]. Similarly, reduced endothelium-dependent constriction observed the first 1-2 weeks post-injury [150, 179], recovers at 1 month [150, 218].

In humans, reduced endothelium-dependent function at 4 weeks [45, 47, 48] typically returns to baseline at 3-12 months [46, 48, 188], whereas VSMC function is generally preserved or appears to recover more quickly, with improved function at 1 month post-catheterization [48, 49, 183]. In addition, marked endothelium-dependent constriction has been reported in denuded radial arteries at 6 months post-PCI, compared to the control vessel. Structural remodeling seems to be more complicated and persistent; with increased IMT still reported 1-4 months post-PCI [169-171] (Figure 2.2).

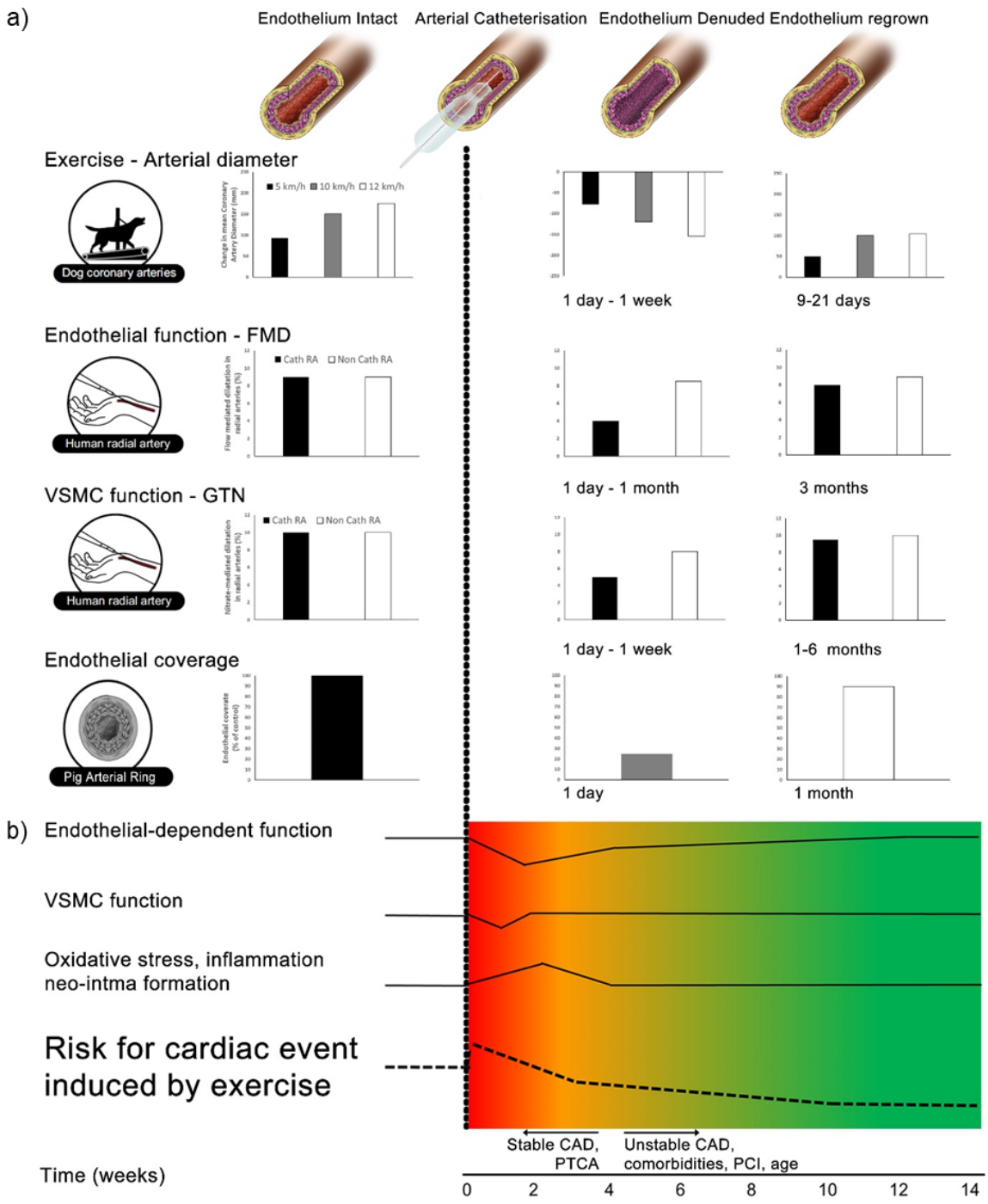
EPC derived from bone marrow have been proposed as a source in endothelial regeneration following vascular injury [219-222]. EPC have been shown to decrease in count [223] and function [224] following PCI. In addition, Gao *et al.* [225] indicated that EPC mobilization depending on the degree of arterial injury post-PCI and from other clinical factors (BMI, smoking), whereas other studies reported no association between EPC and the recovery of vessels [48] or re-endothelialization or reduce restenosis rate [64, 226]. Therefore, further research is needed to define the role of EPC in vascular recovery post coronary intervention. Alternatively, following

endothelial denudation, re-endothelialization begins to cover and repair the denuded area. Endothelial repair is predominantly a result of replication and migration of intact adjacent non-injured ECs, close to the denuded area. In support of this, endothelial repair tends to begin from the edges of the denuded zone (close to intact endothelium) and then converge on the center [227-230]. Although EPC “boosting” thorough drugs or when employed to stents may be an attractive solution towards endothelial recovery post-denudation, the study of EPC mobilization is challenging. Consequently, questions regarding a) the effect of PCI on EPC count and function and b) whether EPC count/function is associated with lower restenosis rate, have still remained unsolved [231].

Regenerating endothelium appears to re-establish the capacity for arterial constriction before the ability to induce dilation [218]. This may result in a greater long-term contractile capacity, increasing the risk for vasospasm and thrombotic events. Indeed, paradoxical vasoconstriction in coronary arteries (treated and non-treated) has been reported [42, 232] at 1 to 6 months post-PCI. Interestingly, incomplete re-endothelialization is apparent at 1-month post-PCI only in DES-treated arteries, while complete recovery is reported in arteries treated with balloon-inflation only or BMS [150]. DES induces non-specific anti-proliferative effects; not only reducing the VSMC proliferation rate but also influencing the growth of other cell types, including ECs. It may negatively affect the recovery of the endothelium post-denudation.

Endothelial regeneration post-injury is associated with the incidence of thrombotic events [17], supporting the importance of early re-endothelialization. Delayed recovery

may be associated with endothelial dysfunction [233, 234], explaining in-stent restenosis [157, 158] or late thrombosis [159, 160], post-PCI. However, it is important that the endothelial lining functions well in order to reduce thrombosis and restenosis risk [64, 185, 235, 236]. Even in arteries with intact endothelial coverage [186], functional abnormalities may occur and are related to intimal thickening [174, 177, 237, 238]. Promoting functional re-endothelialization may be an important strategy to eliminate adverse complications following coronary interventions; however further research is required to understand the cellular and molecular mechanisms that drive ECs to induce endothelial regeneration.



<u>Figure 2.2.</u> Time-course of arterial recovery following coronary interventions. Adapted from Figure 4 (Green *et al.* 2017) [25]. a) Summary of the outcomes of studies that investigated the effects of catheterization in arterial function and structure. Paradoxical vasoconstriction reported in *canine* denuded coronaries in response to

exercise 3 days post-catheterization, and recovered at 9 days [15]. Given the lack of human data for coronary endothelial dysfunction post catheterization, which is the most relevant for the risk of cardiac events, we included information from catheterized radial arteries, as a useful surrogate for coronary arteries. Endothelial dysfunction, assessed by flow mediated dilation (FMD), reported in patients' catheterized radial arteries 1 day to 1 month post-catheterization and tend to recover at 3 months [46, 48, 188]. Vascular smooth muscle cell (VSMC) function generally recovers more quickly; in humans reduced VSMC function in radial arteries is apparent only the first week post-denudation [48, 49, 183]. Endothelial coverage in pig's arterial rings was 25% at 1 day post-catheterization, when compared to the control-uninjured vessel, and recovered up to 80% at 1 month [177]. b) Optimal time to onset exercise training post coronary interventions (PTCA: percutaneous transluminal coronary angiography, PCI: percutaneous coronary intervention). We proposed that patients who undertake PTCA and/or PCI should safely begin exercising at 4 weeks. Stable coronary artery disease (CAD) patients who undergo only PTCA may be able to start exercise training between 2-4 weeks post-PTCA, at a hospital setting, under supervision. Worth noting that oxidative stress, inflammation markers and neo-intima formation which related to the denudation [195-202], follows the same time pattern; increase immediately post denudation [199], remaining elevated for hours [197] or even days (1-15 days) [195, 201, 209, 210], and returning close to baseline levels at 1-month [211].

2.4. Can exercise limit the detrimental impacts of catheterization or artery function and structure: Generalized impacts of exercise training on vascular function and health.

Traditionally, the impact of exercise training in CAD patients has been focused on improvements in fitness levels [33, 37, 239, 240] and cardiovascular risk factors, such as hypertension and lipid profiles [37, 241]. However, this cannot fully explain the exercise-induced cardiovascular risk reduction [241, 242]. It has been suggested that this 'risk-factor gap', can be explained through exercise-mediated improvements in factors such as endothelial function [25, 243]. Indeed, there is compelling evidence that exercise in CAD patients directly improves vascular function [26-37], in exercised limbs [28], non-exercised limbs [37, 244, 245] and in coronary arteries [246], all of which contribute to reduced ischemic events [247]. This is found in traditional aerobic-based training sessions, combined circuit training with resistance exercise [244, 248] and high intensity exercise training (HIIT) [37].

The impact of exercise training can be profound. Exercise training has been shown to have superior outcomes at 1 year (event-free survival, re-hospitalization, and repeat revascularizations) in CAD patients, compared with those who had PCI [143] with reduced inflammation and ischemic events still apparent at 2 years [249]. In contrast, no difference in the progression of *de novo* lesions [250] and plaque formation [251] have been found among exercised and non-exercised groups, although this may be related to efficacy of drug treatment and/or the short time-course for the development of atherosclerosis. However, exercised patients exhibited greater improvements in recurrent angina and maximum exercise tolerance than non-exercised patients [252].

More importantly, exercise training has been associated with lower 5-year all-cause mortality in post-PCI patients [38] and is recommended as a key treatment in CAD [22].

It is worthy of note that CAD patients are typically prescribed a range of CVD medications, including statins [253, 254], beta-blockers [255], angiotensin converting enzyme inhibitors and/or angiotensin receptor blockers [256-259], and sometimes calcium channel blockers [260], all of which may positively impact upon the endothelial function. Few studies have directly assessed the combined impacts of exercise and CV medications on endothelial function, but Walsh *et al.* (2003) showed that basal NO bioactivity was increased following exercise in both treated (with statins) and untreated subjects, suggesting that exercise may benefit vascular function independently of the impact of statins per se [244]. To further support this, large-scale meta-analysis comparing medical treatment with and without exercise training, reported that the combination contributes to significantly lower cardiovascular risk and mortality [261, 262], indicating that some of the direct effects of exercise may be due to the impacts of shear stress and hemodynamics [243]. In conclusion, CVD medication and exercise may have independent effects on vascular function, mediated through distinct pathways that may culminate in synergistic enhancement in endothelial function.

2.4.1. Mechanisms by which exercise training leads to vascular adaptation. The role of shear stress.

During exercise, metabolic demand of skeletal muscle increases drastically. Consequently, skeletal muscle blood flow raises to match the delivery of oxygen to the

metabolic demand [263]. Indeed, skeletal muscle blood flow is closely coupled to metabolic demand [50, 264], [265]. Blood flow is depended on vascular resistance and blood pressure. Blood pressure is increasing during exercise due to activation of sympathetic system, therefore, vascular resistance requires to drastically decline to facilitate adequate blood flow in exercised skeletal muscle [266]. The overall regulation of skeletal muscle blood flow is achieved through a balance between, sympathetic vasoconstriction and vasodilators derived from skeletal muscle [50]. Arterioles are small-diameter resistance vessels and are primary site of both vascular resistance and regulation of blood pressure. Rapid release of local vasodilators in skeletal muscle arterioles at the onset of exercise leads to reduced vascular resistance and increased flow, which may trigger a vasodilator response to flow-mediated arteriolar vasodilation and resultant conducted vasodilation in the upstream vessels at a feed-forward manner [267]. Indeed, arteriolar vasodilation of skeletal muscle enables increased oxygen perfusion in the skeletal muscle tissue [268], whereas impairment in arteriolar vasodilation previously observed in elderlies [269] or hypertensive and diabetic patients [270] resulted in lower vascular conductance and limb blood flow, which may affect oxygen perfusion to muscle tissue, leading to ischemia and termination of exercise. Importantly, this rapid increase in blood flow in skeletal muscle arterioles at the onset of exercise results in elevation of shear stress, which mechanically activate endothelial cells to produce vasodilators (See the section 2.4.2 for further details) and therefore sustained increased blood flow during exercise.

In addition to increase in flow at the arteriole level, there is also an acute vasodilation of larger conduit arteries in response to exercise ensures adequate blood flow to skeletal muscle. Importantly, when conduit arteries are exposed to prolonged periods

of increased flow and shear (regular exercise), vessel remodelling can occur, resulting in a larger vessel lumen [25]. Indeed, increase in shear stress is the prominent mechanism through which exercise training leads to vascular adaptation in both arterioles and larger conduit vessels [25, 50, 264, 265]. Shear stress is the tangential force of the flowing blood on the endothelial surface of the blood vessel, and it is known that high shear stress promotes EC survival, enhancing vasodilation. Conversely, low shear stress will result in EC apoptosis and vasoconstriction, along with VSMC proliferation and platelet aggregation [39]. Indeed, larger conduit arteries (carotid and coronary) exposed in low shear stress and/or disturbed or non-laminar blood flow are more likely to develop atherosclerotic plaques [271, 272]. These observations lead to further studies associating low shear stress to impaired endothelium-dependent dilation in response to ACh in human coronaries [273, 274], indicating that endothelial dysfunction is the potential link between low shear stress and atherosclerosis development [275].

To illustrate the relevance of shear stress in terms of exercise-mediated vascular adaptation, studies were performed in healthy people using an inflated cuff on one exercised limb, in order to blunt the shear stress that accompanied exercise compared to the contralateral unimpeded limb. Following 8 weeks of exercise training, the uncuffed (shear stressed) exercised limb exhibited significant improvement of endothelial function when compared with the cuffed limb [25, 276], supporting the proposal, that shear stress is the key stimuli during exercise to improve endothelial function (see recent review [25]). This was further supported by similar study in which shear stress increases induced by passive heating (i.e. in the absence of exercise) induced identical adaptation, with similar levels of attenuation in the cuffed side [41].

However, this link between shear stress and endothelial function has created further questions regarding how ECs sense shear stress and transduce biological signals, and whether this mechanotransduction system is compromised in subjects with endothelial dysfunction (See section 2.4.2). To conclude, increases in shear stress represents a major stimulus for exercise-related vascular adaptations at both macrovascular and microvascular level, which is vital to matching oxygen delivery to oxygen demand in the 'exercising' skeletal muscle.

2.4.2. Endothelial mechanotransduction pathways

Shear stress is sensed through a variety of EC receptors which transmit mechanical forces to recipient molecules through mechanosensitive signalling pathways [57]. *In vitro* experiments using flow systems have shown that EC mechanotransduction can be mediated by multiple mechanosensors, including junctional proteins (VE-cadherin), receptor kinases (vascular endothelial growth factor receptor 2 (VEGFR-2) and others)), integrins, focal adhesions, G-proteins, G-protein-coupled receptors, ion carriers and glycocalyx [57].

Studies on cultured ECs demonstrate that the response to shear stress is mediated by a mechanosensory complex consisting of VEGFR-2, VE-cadherin and platelet endothelial cell adhesion molecule 1 (PECAM-1) [277]. Within the complex activation of PECAM-1 through phosphorylation has been shown to transduce forces to activate Src family kinases, which phosphorylate VEGFR-2 promoting activation of PI3K and phosphorylation and activation of eNOS [277, 278]. In contrast, VE-cadherin functions as an adaptor that interacts with VEGFR-2 [279, 280].

However, in humans, elevation in shear stress, through passive leg movement, did not phosphorylate VEGFR-2, VE-Cadherin or PECAM-1 in skeletal muscle [281]. This contrasts with *in vitro* cell culture models where application of laminar shear stress has resulted in phosphorylation of these proteins [279, 282, 283]. The absence of response demonstrated by Gliemann *et al.* [284] could be due to the use of passive movement as the means to induce elevated shear stress. Firstly, the change in shear stress experienced during passive movement is relatively low compared to cells in culture which were exposed to a large change in flow [281]. As such, it is possible that exposure of ECs *in vivo* to a greater change in shear stress or added metabolic signalling by active exercise, would cause activation of the mechanosensory complex. However, to our knowledge, there is no study investigating the activation of this complex in human ECs following exercise-induced elevations in shear. Therefore, further studies are required to provide information regarding the activation of mechanosensory proteins in response to elevated shear stress in humans.

Other mechanisms.

Mechanisms other than shear stress, such as reduced inflammation and oxidative stress following exercise training, will all contribute to improved endothelial function [31, 193, 285]. This may also be associated with reduced platelet aggregation [286, 287], contributing further to decrease in the risk of thrombotic events [288] and reduced restenosis risk at 6- [31] and 9-month angiographic follow-up [289, 290]. In summary, improved endothelial function following exercise training may be a result of increased eNOS and NO production [30, 291, 292] and decreased oxidative stress [30] and NO scavenging, such that regular physical activity restores the balance between NO production and NO inactivation in CAD [36]. In addition, increased

numbers of EPC following exercise training may be another mechanism by which exercise ameliorates endothelial function in CAD [293]. This is supported by an association in EPC count and improved FMD and NO synthesis [34].

The studies outlined above indicate that there are profound direct and indirect effects of repeated exercise stimulation on the function, structure and health of conduit arteries in humans. Such benefits have been summarized in a recent review [25]. These effects would be expected to enhance the recovery from catheter related injury, but there is scant evidence regarding the most appropriate time to begin a preventive exercise program, or indeed whether pre-rehabilitation prior to catheterization maybe be as beneficial as post hoc training to enhance recovery [294]. Similarly, it is not fully known if systemic exercise can directly impact a localized area of damage. However, there are several studies that have shown systemic exercise can lead to a change in shear stress in non-exercising areas [41, 276] and that systemic exercise is likely to increase coronary artery blood flow and shear stress [15, 295]. Despite the evidence supporting that exercise-based CR benefits the event-free survival of patients post coronary interventions, the participation rate in both Europe and United States is far lower than desirable; approximately one-third of patients participate in cardiac rehab programs after a cardiac event [296-299]. In the United States, CR referral was remarkably lower in post-PCI than post-CABG patients; 48% and 91%, while the hospital performing the procedure was the strongest predictor of referral [300]. Therefore, an increase in referrals should be considered as a priority in CAD management.

2.5. What are the vascular effects of acute exercise after PTCA/PCI?

Whilst regular exercise has clearly associated with improved endothelial function and reduction of cardiac events, the acute response to a single bout of exercise remains a controversial issue, particularly exercise in patients following coronary interventions. It is proposed that strenuous exercise might acutely enhance the risk of events in cardiac patients, including thromboembolism and myocardial infarction [301]. Although such events are extremely rare following maximal systemic exercise; stent thrombosis risk 0-0.02% [302] and 1% [303], some isolated case-reports of sub-total or total occlusion of coronary arteries [301], and fatal acute stent-thrombosis [304-306], have been related with acute systemic high-intensity exercise following coronary interventions. It is worth noting that despite the Pohl *et al.* [16] data indicating vasoconstriction of *canine* femoral arteries post-injury in response to leg exercise, there is no data regarding localized effects of acute exercise in humans' arteries following catheterization (See section 2.5.2 for more details). The dichotomy between the increased acute risk of exercise and the well-established sustained benefits of prolonged exercise training is commonly known as the 'exercise paradox', whereby reduced endothelial function [307] and increased platelet aggregation [308] have been shown in acute strenuous exercise, whereas improved endothelial function has been reported as a result of prolonged exercise training in CAD patients [37].

2.5.1. Endothelial function in healthy and CAD population.

The impact of acute exercise on endothelial function (as assessed using FMD immediately following exercise) in healthy individuals is equivocal; studies showing an increase [309-311], a decrease [312-314], or no change [315-317]. This variation may relate to exercise intensity, type, duration and the subject's fitness level [148]. In

particular, prolonged exercise has been shown to reduce endothelial function in healthy individuals [318] and result in negative effects on vascular stiffness in CAD patients [319]. Despite the duration, exercise intensity (typically defined as the % of maximal heart rate), has been considered as the 'key-factor' driving the vascular responses. In general, high intensity exercise (HIE) causes greater acute endothelial *dys*function than moderate intensity exercise (MIE) [148, 314, 320]. However, the role of exercise intensity on vascular responses in CAD population is unclear, with studies showing greater dysfunction in HIE [321], and others no difference compared to MIE [322-324]. The above controversy may be explained, in part, by the fact that each study had different exclusion criteria to define CAD patients; i.e. including or excluding patients with previous coronary interventions within 3 months. Differences in baseline FMD appears to affect the vascular responses acutely post-exercise; lower baseline FMD may result in increase in endothelial function post-exercise, while higher baseline-FMD may be associated with a decrease [324], further clouding the understanding of acute effects of exercise on endothelial function. Apart from non-invasive FMD data collected post-exercise, more invasive studies in animals have demonstrated an impairment in endothelial function to increased blood flow during exercise, paradoxical vasoconstriction to acetylcholine (ACh), but preserved VSMC dilation in atherosclerotic animals, whilst arteries from healthy animals dilate [325]. Similarly, Gordon *et al.* [326] suggested that vasoconstriction in response to ACh and exercise is apparent only in patients with atherosclerosis, while patients with angiographically smooth vessels appeared to preserve endothelial vasodilation.

Circulating ECs, soluble E-selectin and vWf, (markers of endothelial dysfunction), are all elevated acutely post-exercise stress tests in CAD patients [327], suggesting that

exercise bouts may impact on endothelial function in these patients. To our knowledge, only one study evaluated VSMC function immediately post a single bout of submaximal cycling exercise in CAD patients, showing a decrease at 15min post-exercise [324]. Whilst the above studies have examined peripheral arteries, changes in coronary artery function with isometric handgrip exercise has also been examined in patients with CAD [328, 329]. Isometric handgrip exercise resulted in abnormal coronary responses with reduced vasodilation and blood flow [328], supporting the incidence of coronary endothelium-dependent dysfunction [329].

2.5.2. Endothelial function post-denudation.

Even less evidence exists regarding vascular responses of acute exercise in vessels that have been catheterized (Figure 2.1&2.2). Pohl *et al.* 1986 first illustrated i*n vivo* paradoxical vasoconstriction post-denudation in response to increased blood flow and ACh (endothelial-mediated function) in femoral arteries, whereas dilation appeared to be preserved in response to nitroglycerin (VSMC function) [16]. Following on from this, Berdeaux *et al.* 1994 reported marked vasoconstriction in response to exercise in canine epicardial arteries post endothelial denudation. This 'paradoxical' vasoconstriction in denuded arteries has also been observed following administration of ACh (endothelial-mediated function) and nitroglycerin (VSMC function). VSMC function was restored at 3 days and endothelial-mediated function in response to exercise and ACh at 9 days [15].

More recently, studies in patients performing supine bicycle exercise during coronary catheterization reported an exercise-induced paradoxical vasoconstriction in

coronary-treated artery, at 6 months post-PCI with 1st generation [330], and at 16 months with 2nd generation DES [331], with normal vasodilation in the non-catheterized vessel [331]. VSMC-induced dilation was abolished in the stented area, whereas vasodilation was still apparent proximally and distally to the stent [331]. Coronary vasoconstriction during exercise, 6-months post-PCI, has been implicated in chest-pain with the absence of significant stenosis in a recent case report [234]. Overall, these data suggest that coronary interventions result in impaired vascular responses to acute exercise in the stented coronary arteries, mainly in endothelium-dependent function, which may increase the risk for vasoconstriction, spasm and possibly cardiac events.

Potential mechanisms.

There are a number of different mechanisms underlying both the acute decrease in vascular function and increased platelet aggregation, explaining the risk of exercise-related cardiac events (Figure 2.1). Changes in inflammation and oxidative stress can affect endothelial and platelet function. Inflammatory markers (C-RP, IL-6, IL-8, TNF-a) are increased immediately following HIE, in healthy subjects [308] and CAD patients [332, 333], while no significant inflammation response appears following lower intensity exercise [308]. It is suggested that this acute immune response may stimulate thrombosis by enhancing both platelet activation and endothelial damage [308, 334]. Furthermore, a negative correlation between vascular function and platelet aggregation post-exercise has been reported in CAD [335], but not in healthy subjects [335] or pre-clinical populations [336], suggesting that existing endothelial dysfunction may be associated with attenuated platelet aggregation, resulting in increased

thrombotic risk post-exercise. In addition, strenuous exercise leads to an immediate increase in oxidative stress in CAD patients [337], which appears to promote platelet responsiveness [338] and endothelial dysfunction by reducing NO bioavailability [308]. Vitamin C (an antioxidant) abolished the increased oxidative stress and endothelial dysfunction (FMD) post-exercise in CVD patients [339]. Interestingly, some *in vivo* studies have maintained that anti-oxidant therapy (ascorbic acid or glutathione) results in reduced coronary artery spasm [340], and platelet aggregation [341]. All of these mechanisms appear to follow a similar time-course, with an increase immediately post-strenuous exercise, followed by a normalization.

2.6. Summary

Cardiovascular disease (CVD) is the number one cause of morbidity and mortality worldwide. Although the exact mechanisms leading to each cardiovascular condition depend on the disease in question, endothelial dysfunction and development of atherosclerosis have been observed in all forms of CVD. Endothelial dysfunction is often characterised by a reduction in NO bioavailability, and this may be the result of an imbalance between NO production and NO scavenging by superoxide anions and related reactive oxygen species (ROS). Elevated oxidative status may also enhance the production of vasoconstrictors or pro-inflammatory and senescence markers, contributing further to endothelial dysfunction and atherosclerosis progression.

Presence of atherosclerotic plaques in coronary arteries, known as CAD, has been classified as the number one cause of death among CVD. Diagnosis and treatment of CAD often involve catheterization procedures (PTCA and/or PCI). Such procedures are likely to mechanically damage the endothelial layer, leading to structural and

functional alterations. Indeed, paradoxical vasoconstriction in response to increased flow has previously been observed in animals. Such evidence questions the use of exercise in CAD patients, at least its immediate use after catheterization. Since exercise-based CR is recommended for all CAD patients as it enhances endothelial function and lowers mortality and morbidity, it is important to evaluate the time-course of recovery from the “insult” of catheterization in coronaries, in order to propose the safe onset of exercise training following catheterization. However, given the inherent risk and invasive nature of evaluating coronary artery function following catheterization, less invasive procedures in the periphery, such as radial artery function have been suggested, as both arteries are comparable in histopathology and size [44].

Elevated shear stress has been proposed as the predominant mechanism behind the atheroprotective benefits of exercise. Although there is clear evidence demonstrating a beneficial role of shear stress in endothelial function, little is known about how ECs sense and transduce shear stress and how this leads to vascular adaptation. PECAM-1, VE-Cadherin and VEFGR-2 have been activated by increased shear in EC culture models, whereas there is only one study in humans (in the muscle microcirculation), reporting no activation of such proteins. Given this dichotomy and the limited information regarding the mechanosensory system in general, further studies are required to explore the mechanisms by which ECs sense shear. This will help us to better understand the beneficial role of exercise in cardiovascular disease.

CHAPTER 3 - General Methods

3.0. Experimental procedures

Experimental procedures were conducted in two locations. For the studies descripted in **Chapter 4 and 5**, experimental visits were conducted in the Exercise Physiology Room (Room 3 Hawthorn suite) at Liverpool Heart and Chest Hospital (LHCH). For studies in Chapter 5 and 6, all experimental procedures took place in temperature-controlled rooms (~22^{o}C) at Liverpool John Moores University (LJMU).

In all studies, during the first visit, participants had their height measured using stadiometer (Model 20, Seca, Germany (LJMU) or Leicester Height Measure, Germany (LHCH)) and weight measured using digital scales (Model 767, Seca, Germany (LJMU) or Accuweight Skidproof Digital Body Weight Scale, UK (LHCH)). Body mass index (BMI) was retrospectively calculated using the equation [weight (kg) / height (m^2)].

3.1. *In vivo* vascular assessments

The present PhD thesis includes two methods to assess function of peripheral arteries: a) flow-mediated dilation (FMD), and b) vascular responses (i.e. arterial diameter, blood flow) to handgrip exercise (HE). These vascular assessments were used in **Chapters 4-6 and 4, 5 and 7**, respectively. The methods used in these Chapters were identical and are descripted in detail below.

Prior to vascular assessments, subjects were instructed to abstain from alcohol, vitamins, drugs and exercise for 24 hours, and be fasted (including caffeine) for at least 6 hours prior to each visit, in accordance with most recent guidelines [342, 343].

3.1.1. Flow-mediated dilation (FMD)

This test was first introduced by Celermajer *et al.* in 1992, to non-invasively assess endothelial function in peripheral arteries in response to hyperaemic stimuli [344]. Since then it has evolved and now strict guidelines are provided to aid reproducibility and reliability [342, 343]. Briefly, the experimental artery is imaged using non-invasive Doppler ultrasound. A pneumatic cuff is then inflated, and released, providing a hyperaemic stimulus. This elevated shear stress in the artery activates mechanoreceptors in ECs, to subsequently release vasodilators and increase arterial diameter [345]. Is has been suggested that FMD response is primarily mediated by endothelial NO production [346]. Therefore, with respect to the proposed PhD thesis, FMD has been used to assess endothelium-dependent NO-mediated dilation, following different experimental interventions.

Coefficient of variation for the sonographer in regards to FMD was tested as follows. Ten young healthy individuals requested to attend the CV lab at LJMU on two different occasions (>72 hours and <2 weeks between visits) to perform one brachial FMD. Subjects were prepared following the aforementioned guidelines, and FMD was performed and analysed in accordance with standardised guidelines [342, 343]. Coefficient variation was 6.14% and 4.06% for brachial FMD and baseline artery diameter respectively.

In this PhD thesis **(Chapters 4-6)**, FMD was measured in the radial artery in the lower arm. Participants rested in the supine position for >10 minutes before measurement, to ensure that all hemodynamic variables were stabilised. Blood pressure (BP) and heart rate (HR) were measured using an automated sphygmomanometer (GE Pro

300V2, Dinamap, Tampa, FL, USA). At least 5 minutes were given between BP measurements and the onset of FMD, to ensure that BP cuff inflation did not affect the FMD response.

In **Chapter 5**, FMD was undertaken in a single arm (the catheterized arm), whereas in **Chapters 4 and 6**, FMD was undertaken simultaneously in both arms (bilateral FMD). Here we describe the protocol for a single arm. In bilateral FMD, the same equipment and protocol was used, and measurements were taken simultaneously in both wrists.

To assess radial artery (RA) FMD the arm was extended ~45° from the torso, and an inflation/deflation pneumatic cuff (D.E. Hokanson, Bellevue, WA) was placed proximal to the wrist to provide a stimulus for forearm ischemia. Two 12-MHz multi-frequency linear array probes, attached to high-resolution ultrasound was used to image the radial artery 10-15 cm proximal to wrist (Figure 3.1). Once an optimal image was obtained, the probe was held stable and the ultrasound parameters were set to optimize the longitudinal, B-mode image of the lumen–arterial wall interface. Continuous Doppler velocity assessments were also obtained using the ultrasound and were collected using the lowest possible insonation angle (always $< 60°$). Following baseline measurement (1 minute), the forearm cuff was inflated (> 200 mmHg) for 5 minutes. Diameter and flow recordings resumed 30s prior to cuff deflation and continued for 3 minutes thereafter, in accordance with technical specifications [342, 343].

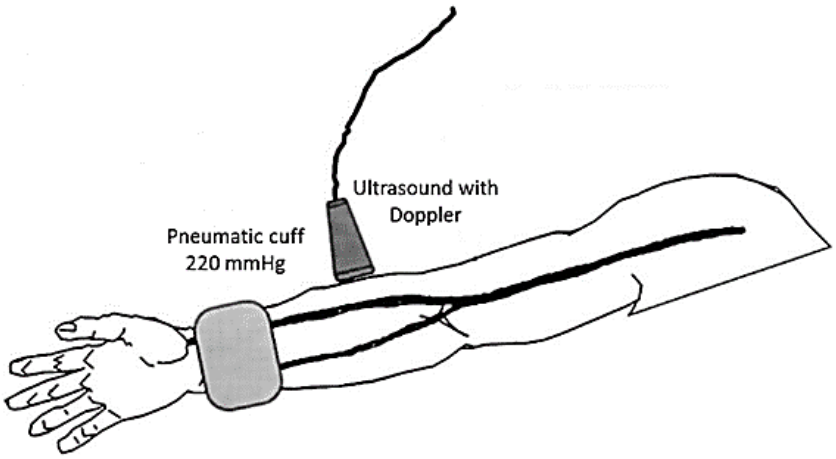


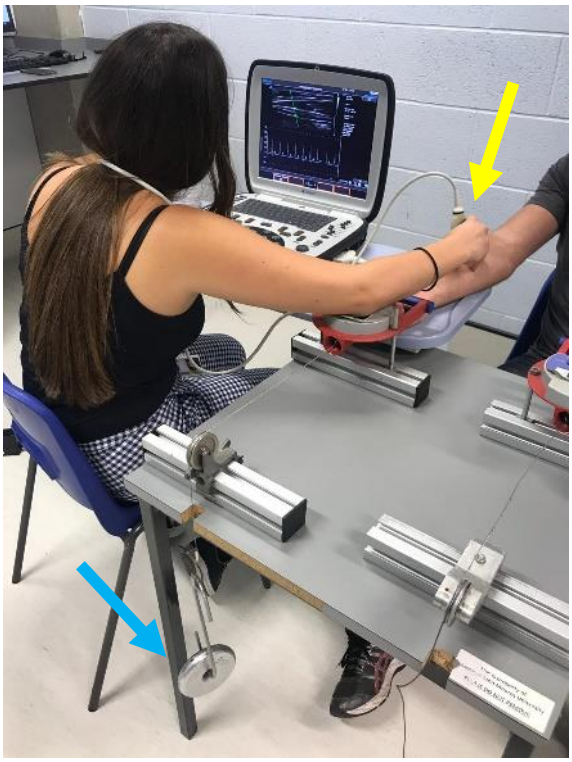
Figure 3.1. Schematic illustration of radial artery (RA) flow-mediated dilation (FMD).

A pneumatic cuff was placed around the wrist and Doppler ultrasound was used to image the RA proximal to wrist. For detailed information in regards to radial artery FMD, see section 3.1.1. Adopted from [347]

3.1.2. Vascular responses to handgrip exercise (HE)

In this PhD thesis **(Chapters 4, 5 and 7)**, vascular responses to HE was measured in the radial artery in the lower arm (10-15 cm from the wrist). In **Chapters 5 and 7**, HE responses were measured in a single arm (the catheterized arm), whereas in **Chapter 4,** HE responses were assessed in both radial arteries simultaneously (bilateral HE). Here we describe the protocol for a single arm. In bilateral HE, the same equipment and protocol was used, and measurements were taken simultaneously in both wrists. A 12-MHz multi-frequency linear array probe, attached to high-resolution ultrasounds was used to image the experimental artery. The Experimental arm were rested on a table in order to remain as stable as possible (Figure 3.2). The HE protocol used was

different between studies; relevant information is available in each Chapter. Arterial diameter and blood velocity recordings were obtained prior to HE and during or at the end of each exercise, depending on the study protocol; clarification is provided in each Chapter. An automatic metronome (Korg MA30 Metronome 2006, Japan), was used to keep a constant pace for the HE (30 contractions per minute).



<u>Figure 3.2.</u> Method of assessing radial artery (RA) responses to handgrip exercise (HE).

A handgrip table was used, where weights - equal to the 5%, 10% or 15% of pre-estimated maximal voluntary contraction (MVC) (depending on study protocol), were added to hooks connected to handgrip (blue arrow). This allowed participants to perform HE at a specific, constant intensity according to the study protocol. The arm

was rested stable on the table, and the sonographer scanned the RA (yellow arrow) before and after the HE bout, using non-invasive Doppler ultrasound.

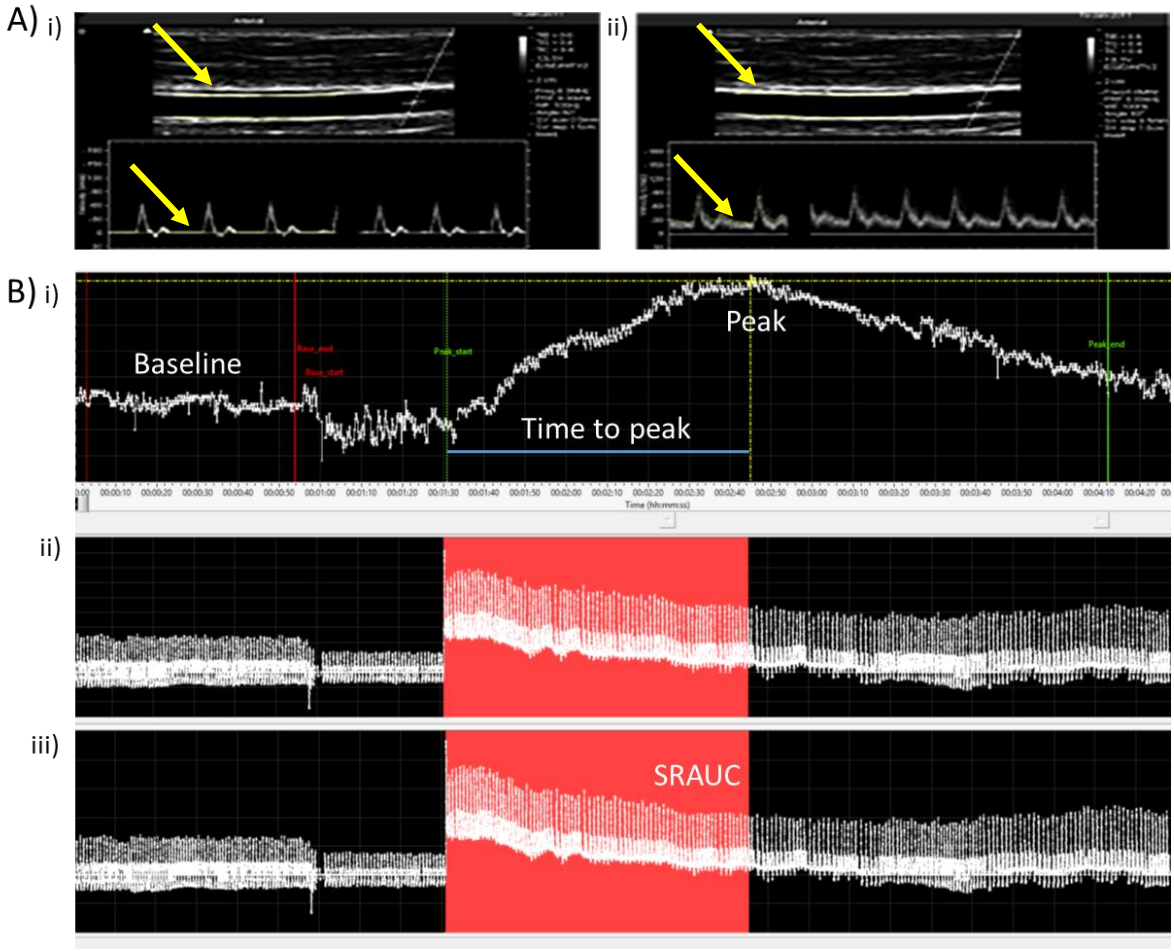
3.1.3. Data analysis (FMD and response to HE)

Custom-designed edge-detection and wall-tracking software was used to analyse both the FMD and arterial diameter and blood flow changes in response to HE, in order to minimise the investigator bias [342, 348]. The same software was used in all Chapters. An optimal region of interest to be analysed was selected by the sonographer, chosen to obtain a clear distinction between the artery walls and lumen.

FMD was reported as the maximum percentage change in artery diameter from baseline to peak when the cuff was released [343]. The software automatically calculated the relative diameter change, time to peak (following cuff release) and shear rate area under the curve (SRAUC) [343] (Figure 3.3).

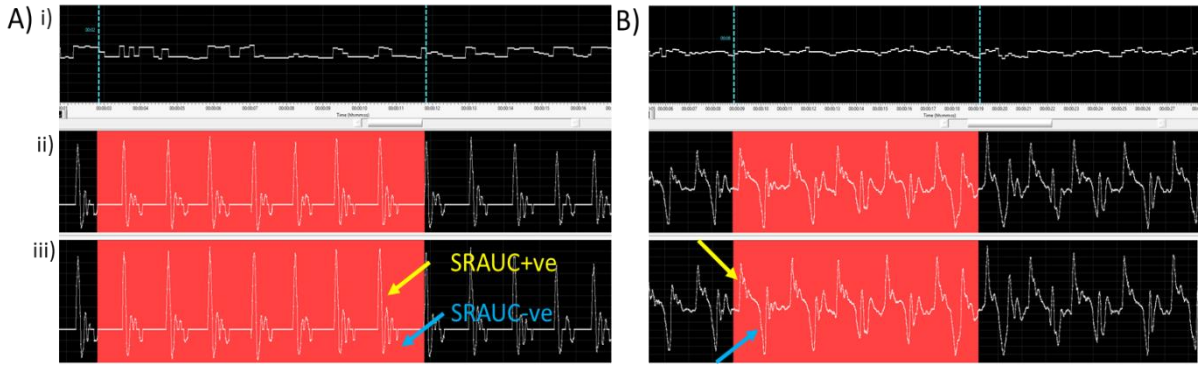
For the vascular responses to HE, each recording lasted approximately 1 minute. The software then automatically calculated the mean of this recording for the following parameters: diameter, blood velocity, total blood flow area under the curve (AUC), anterograde blood flow (AUC+ve flow), retrograde blood flow AUC (AUC-ve flow), mean shear rate (SR), total anterograde SRAUC (SRAUC+ve) and total retrograde SRAUC (SRACU-ve) (Figure 3.4). Mean SRAUC+ve and SRAUC-ve were then calculated as the total SRAUC+ve/60s and SRAUC-ve/60s respectively. As the software was not able to automatically calculate the relative change in diameter to HE, separate recordings were taken at baseline (rest, before HE) and during HE, and

percentage change was manually calculated. Statistical analysis was undertaken to examine such changes. Relevant information is available in each Chapter.



<u>Figure 3.3.</u> Screen shots from flow-mediated dilation (FMD) analysis software.

A) Selection of regions of interest (see yellow arrows) for diameter and blood velocity, at baseline (i) and after hyperemic stimuli (ii). B) Automatic calculation of the FMD, as a percentage change between baseline diameter, and peak diameter during hyperemia stimuli (i). Time to peak (i) (blue line), was automatically calculated, from when the cuff was released until peak diameter was reached. Blood velocity (ii) and SRAUC (with red) (iii), during the FMD were also calculated automatically by the software [343].



<u>Figure 3.4.</u> Screen shots from handgrip exercise (HE) analysis, using the custom-designed edge-detection and wall-tracking software.

A) Rest (before HE) and B) during HE. Diameter (i), blood velocity (ii) and shear rate (SR) (iii) were calculated automatically by the software. Arrows indicate anterograde SR, area under the curve (SRAUC+ve) (yellow) and retrograde SR, area under the curve (SRAUC-ve) (blue), which were also calculated automatically.

3.2. Exercise performance assessment

3.2.1. Aerobic capacity test (Chapter 7)

This test was used to measure the peak oxygen consumption (VO_{2peak}) during maximal graded exercise. Participants completed an incremental exercise test to exhaustion on an electromagnetically braked cycle ergometer (Lode Excalibur Sport Cycle Ergometer, The Netherlands) to determine the $VO_{2peak,}$ using an online gas collection system (MOXUS Metabolic Cart (AEI Technology, USA). Briefly, participants started cycling at 60W for 3 minutes; following this the workload was increased by 35 W every 3 minutes until volitional fatigue. VO_{2peak} corresponds to the highest value achieved

over a 15s recording period. Participants were instructed to avoid eating ≥3 hours and exercise ≥24 hours prior to the test.

3.2.2. Handgrip strength test (Chapters 4, 5 and 7)

Maximal voluntary contraction (MVC) was measured using a standard dynamometer (manual muscle strength gauge gripped with the whole hand) (Takei 5420 Grip-D Digital Hand Grip Dynamometer, Japan). Participants held their arm straight up in the air (12 o'clock) and in one continuous movement, squeezed the dynamometer as hard as they could while moving their straight arm 180 degrees until it was by their side. This test was performed on both arms. The highest value achieved on 3 attempts was used to determine the MVC of each arm. MVC was used to calculate the exercise intensity for the following acute HE protocols, depending on the study design.

3.3. Pilot study to examine the vascular responses to incremental HE in young healthy males.

Rationale: The studies descripted in **Chapters 3 and 4** involve the assessment of RA response to incremental HE in coronary artery disease (CAD) patients. Previous work in healthy males has demonstrated that 30 minutes of HE at 5%, 10% and 15% of MVC resulted in increased blood flow and diameter in the brachial artery [309]. Such responses increased within the first 5 minutes of exercise and then plateaued, in all 3 HE intensities, suggesting that shorter exercise bouts may be adequate to track the vascular responses to exercise. However, to our knowledge, there is no information on the RA responses to HE, and whether these are different to larger conduit arteries such as the brachial artery. Therefore, the aim of this pilot study was to examine

whether 3 minutes bouts of incremental HE at 5%, 10% and 15% of MVC were adequate to stimulate increases in blood flow and diameter in the RA of healthy males.

Methods: Twelve healthy young males were recruited (23.1±4.9 years, 24.9±3.2 kg/m^2). Participants were free of known CVD or metabolic disease, non-smokers and abstained from any type of medication or supplements. Informed consent was gained from all subjects prior to the experimental procedures. This study was approved by the Liverpool John Moores Ethics Committee, adhered to the Declaration of Helsinki and met the ethical standards of the International Journal of Sports Medicine [349].

Participants attended the cardiovascular lab at LJMU for one visit, which lasted approximately 30-40 minutes. Participants were fasted for at least 6 hours (including caffeine) and abstained from alcohol, vitamins, drugs and exercise for 24 hours before the visit [342]. MVC was measured in the experimental arm (right) as descripted above. Participants then rested in the seated position for 10 minutes, before BP and HR were measured in the left arm. Subsequently, an incremental HE protocol was performed which was consisted 3 x 3-minute bouts of exercise at intensities of 5%, 10% and 15% of pre-estimated MVC, with 1-minute rest between the bouts (Figure 3.5). RA diameter and blood velocity were measured, as descripted above, before the HE (baseline) and during the 1-minute resting period at the end of each HE intensity stage. An automatic metronome (Korg MA30 Metronome 2006, Japan), was used to keep constant pace for the HE (30 contractions per minute). BP and HR were measured immediately post the HE protocol. A mix-linear model (SPSS 25) was used to compare differences in variables to incremental HE. Statistical significance was set to $P \leq 0.05$. Results are reported as mean±SD.

Results: Haemodynamic measurements, pre- and post-HE, MVC, and RPE at each HE intensity are reported in Table 3.1. Vascular responses to incremental HE are reported in Table 3.2. Example screen shots of artery diameter and velocity in response to HE at 5%, 10% and 15% MVC are shown in Figure 3.5.

Importantly, RA diameter was increased at all HE intensities when compared with baseline (main effect $P<0.001$). RA diameter at 15% MVC was significantly higher than 5% ($P=0.001$) and 10% ($P=0.01$). However, the increase in RA diameter was not stepwise with exercise intensity as no statistical difference was reported between 5% and 10% of MVC ($P=0.368$) (Figure 3.6).

In contrast, there was a significant main effect ($P<0.001$) of exercise intensity with a stepwise increase in blood velocity, total AUC blood flow, anterograde (AUC+ve flow), retrograde flow (AUC-ve flow), mean shear rate, anterograde SRAUC (SRAUC+ve), mean retrograde SRAUC (SRAUC-ve) in healthy individuals (Table 3.2). Indeed, most of the aforementioned outcomes demonstrated a stepwise increase with HE intensity, apart from the retrograde flow and retrograde SRAUC, for which responses were only higher from baseline.

Table 3.1. Hemodynamic measurements, handgrip strength and rate of perceived exertion (RPE), before handgrip exercise (HE), (PRE) and after HE (Post).

	SBP (mmHg)	DBP (mmHg)	MAP (mmHg)	HR (b/min)	MVC (kg)	RPE: 5% MVC	RPE: 10% MVC	RPE: 15% MVC
Pre	122±7	68±17	86±11	67±13	46.8±6.8	2.0±0.9	4.0±1.7†	5.0±1.9†‡
Post	125±7	73±11	90±8	69±11				

SBP: systolic blood pressure); DBP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate; MVC: maximum voluntary contraction; RPE: rate of perceived exertion; scale 1-10 (1: no effort to 10: maximal effort). Results reported as mean±SD, n=12, $P<0.05$, †Significantly different from 5% MVC, ‡Significantly different from 10% MVC.

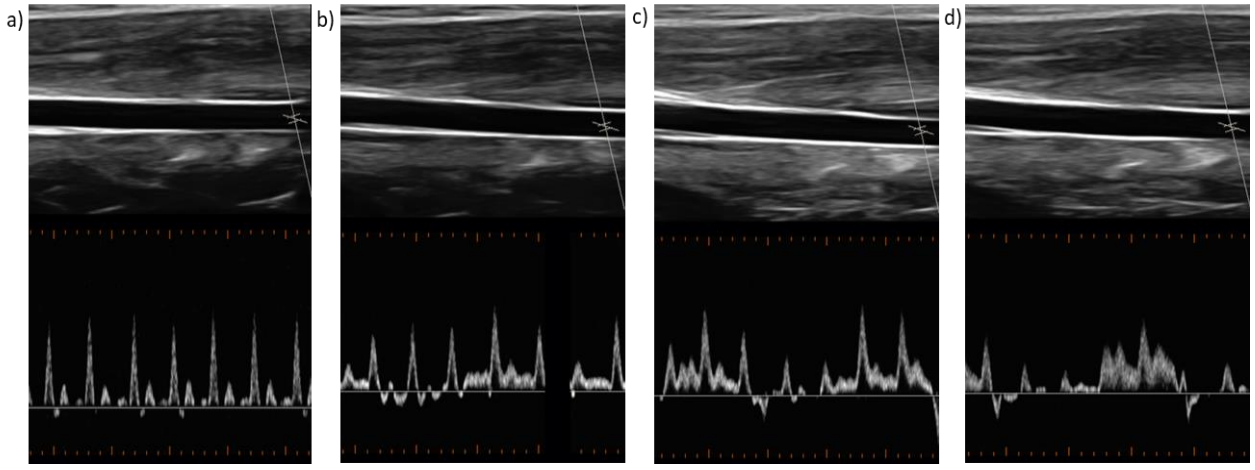
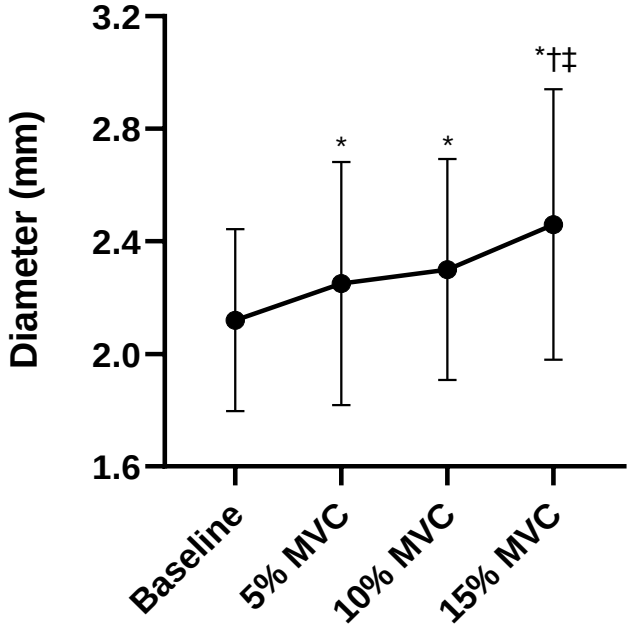


Figure 3.5. Screen shots from measuring vascular responses, using non-invasive Doppler ultrasound at a) before handgrip exercise (HE), b) 5% maximum voluntary contraction (MVC), c) 10% MVC, and d) 15% MVC.

Table 3.2. Vascular responses before handgrip exercise (HE) (Baseline), at 5% of pre-determined maximal voluntary contraction (MVC), at 10% MVC, and 15% MVC.

	Baseline	5% MVC	10% MVC	15% MVC
Blood velocity (cm/s)	4.9±2.5	12.5±7.0*	20.5±8.6*†	26.1±10.4*†‡
Total mean AUC Flow (ml/min)	10.9±5.5	33.7±26.1*	55.6±33.2*†	82.4±49.5*†‡
AUC+ve flow (ml/min)	12.9±6.1	34.3±25.9*	55.9±33.0*†	82.5±49.5*†‡
AUC-ve flow (ml/min)	-2.0±2.6	-0.6±1.2*	-0.3±0.5*	-0.0±0.1*
Mean Shear rate (1/s)	92.1±54.0	220.6±104.0*	356.2±130.5*†	424.5±132.9*†‡
SRAUC+ve (1/s)	108.9±56.0	225.6±101.5*	358.0±128.4*†	424.7±132.5*†‡
SRAUC-ve (1/s)	-16.8±22.1	-5.1±10.1*	-2.0±4.3*	-0.3±1.1*

AUC: area under the curve; AUC+ve: anterograde; AUC-ve: retrograde; SRAUC: shear rate area under the curve; SRAUC+ve: anterograde shear rate; SRAUC-ve: retrograde shear rate; MVC: maximal voluntary contraction. Results reported as mean±SD, n=12, P<0.05, *Significantly different from Baseline, †Significantly different from 5% MVC, ‡Significantly different from 10% MVC.



<u>Figure 3.6.</u> Radial artery (RA) diameter before handgrip exercise (HE) (Baseline), and during the 1-minute recovery at the end of 5% maximum voluntary contraction (MVC), 10% MVC, and 15% MVC.

Results reported as mean±SD, n=12, *P*<0.05. *Significantly different from Baseline, †Significantly different from 5% MVC, ‡Significantly different from 10% MVC.

<u>Conclusion:</u> This pilot study indicates that 3-minute bouts of incremental HE at 5%, 10% and 15% of MVC resulted in stepwise increases in blood velocity, blood flow and mean shear rate in healthy individuals. Arterial diameter was higher in all three HE intensities, when compared to baseline. Importantly, arterial diameter at 15% MVC was significantly increased when compared to 5% and 10% MVC, suggesting the possibility of stepwise increase in diameter, and the intensity changes from 5% to 10% MVC may be not adequate to cause change in diameter. These results illustrated that this specific incremental HE protocol is adequate to evaluate the vascular responses

to acute exercise, and therefore, it can be successfully used for further studies **(Chapter 4, 5 and 7).**

3.4. Transradial catheterization, endothelial cell (EC) collection and EC isolation

EC collection, isolation and protein expression analysis, were descripted originally by Colombo *et al.* [350] and Feng *et al.* [351]. Different equipment and procedures were used for transradial catheterization, endothelial cell collection and isolation between Chapters; two studies **(Chapters 4 and 5)** were conducted in CAD patients at LHCH, while the others **(Chapters 6 and 7)** were conducted in healthy individuals at LJMU, therefore the individual protocols are descripted below. The method to assess EC protein expression was identical between studies and is descripted below.

3.4.1. Cardiac transradial catheterization and endothelial cell (EC) collection in CAD patients at LHCH

*This catheterization procedure is referred to in **Chapters 4 and 5.***

PTCA and/or PCI was performed predominantly via the right radial artery. Local anaesthesia was achieved with 2% lignocaine (Antigen Pharmaceuticals, Ireland). The radial artery was punctured with a 21-gauge arterial needle through which a 0.0118”platinum-tipped nitinol guide wire was introduced. Then, a 5F, 6F or 7F hydrophilic sheath introducer (sheath length 16 cm) was inserted (PreludeEase, MeritMedical, UK). A weight-adjusted dose of heparin and routine use of vasodilator cocktail (nitroglycerine, verapamil, or diltiazem) was introduced into the central

circulation during the procedure as required to eliminate complications such as radial occlusion and thrombosis (see Chapters for more details). Once the procedure finished, researchers collected the J-shaped guidewire (3mm J TEF 150cm x 0.35", KIMAL, UK) and transferred it immediately to a dissociation solution (~30ml) (0.5% bovine serum albumin, 2 mmol/L EDTA, and 100 ug/mL heparin in Dulbecco's Phosphate Buffered Saline (DPBS)). An additional sterile J-shaped guidewire was advanced into the radial artery, ~3-4cm above the sheath, run back and forth to collect ECs from the inside of the RA before being retracted, and transferred immediately to the dissociation buffer. All introducer sheaths were removed at the end of the procedure and haemostasis achieved in the catheterization laboratory through a compression device (MedPlus, UK). The patients were mobilized immediately, while in the hospital until the compression device was removed after ~4 hours.

3.4.2. Endothelial cell (EC) insolation protocol in CAD patients at LHCH

*This procedure is referred to in **Chapter 5**.*

Briefly, the two J-shaped guidewires, collected during the catheterization, were clasped with a pair of forceps and rinsed in dissociation buffer solution for 10 minutes, to gently remove ECs from the wires and transfer to solution. The dissociation buffer solution was then centrifuged at 400 RCF at 4°C for 7 minutes. Subsequently, the supernatant solution was removed, without disturbing the pellet in the bottom. The reminding sample was fixed with 1ml of 3.7% formaldehyde solution for 10 minutes. 15ml of Dulbecco's Phosphate Buffered Saline (DPBS) (Thermo Fisher Scientific, REF number: 14190086, USA) was added and the pellet was resuspended before the

sample was centrifuged at 400 RCF, 4°C for 5 minutes. Subsequently, the supernatant solution was removed, without disturbing the pellet and then 12ml of DPBS was added, the pellet was resuspended in the solution and centrifuged at 400 RCF, 4°C for 6 minutes. Finally, the supernatant solution was removed, without disturbing the pellet (~2ml), resuspended and evenly spread onto 8 glass slides (VWR, REF number: 631-0705, USA) (~250ul each). Slides were dried at 37°C for 1 hour before being stored at -80 °C until further immunofluorescence analysis.

3.4.3. Transradial catheterization and endothelial cell (EC) collection in healthy males at LJMU

*This catheterization procedure is referred to in **Chapters 6 and 7**.*

An 18 or 20-gauge catheter (with 10 and 8cm length respectively (leadercath, Vygon, UK) was placed in the RA of one arm (usually the right arm), under local anaesthesia (Marcaine Polyamp Steripack 0.5%, Aspen, UK) (Figure 3.7). Two separate J-shaped guidewires (paediatric J-wires, 0.46mm, 40cm length, Vygon, UK) were advanced ~3-4 cm beyond the tip of the catheter. The J-wires were run back and forth to collect ECs from the inside of the RA and then retracted. The distal portion of each J-shaped guidewire was transferred immediately to pre-cooled (4°C) dissociation buffer (~30ml, 0.5% bovine serum albumin, 2 mmol/L EDTA, and 100 ug/mL heparin in Dulbecco's Phosphate Buffered Saline (DPBS)). ECs were kept at 4°C and immediately taken to the laboratory for processing. EC collection was repeated immediately after the completion of the forearm exercise protocol or control period. ECs were collected using the same catheter pre and post intervention. The catheter was then removed,

and haemostasis achieved by applying manual pressure. No weight-adjusted dose of heparin or other vasodilators (nitroglycerine, verapamil, or diltiazem) was introduced into the central circulation during the procedure.

Figure 3.7. Catheterization via radial artery, in healthy young participant at LJMU.

This procedure refereed to ***Chapters 6 and 7.***

3.4.4. EC insolation protocol in healthy individuals at LJMU

This procedure is referred to in ***Chapter 7.***

The Falcon tube with the dissociation buffer (˜30ml) and the two J-shaped wires was centrifuged at 400 RCF, 4°C for 7 minutes. The supernatant solution was then removed (leaving ˜2ml and the remaining sample was fixed with 1ml of 3.7% formaldehyde solution for 10 minutes. 15ml of Dulbecco's Phosphate Buffered Saline (DPBS) (Thermo Fisher Scientific, REF number: 14190086, USA) was added and the

pellet was resuspended before the sample was centrifuged at 400 RCF, 4°C for 5 minutes. Subsequently, the supernatant solution was removed, 12ml of DPBS was added and the pellet was resuspended in the solution. The wires were then clasped with a pair of forceps and rinsed in dissociation buffer solution for 10 minutes, to gently remove ECs from the wires and transfer into solution. The sample was centrifuged at 400 RCF, 4°C for 6 minutes. Finally, the supernatant solution was removed, without disturbing the pellet (~2ml), resuspended and evenly spread onto 8 glass slides (VWR, REF number: 631-0705, USA) (~250ul each). Slides were dried at 37°C for 1 hour before being stored at -80 °C until further immunofluorescence analysis.

3.5. Quantitative immunofluorescence

The following procedures are the same and referred to in ***Chapters 5 and 7.***

3.5.1. Staining protocol

Samples were defrosted at room temperature for 5 minutes and rehydrated with DPBS for 10 minutes. slides were then incubated with 5% donkey serum (D9663, Merck, UK) for 1 hour at room temperature to block nonspecific binding sites. Following blocking, slides were incubated with primary antibodies against the proteins of interest (Table 3.3) for 1 hour at room temperature. Cells were also stained for vascular-endothelial (VE)-cadherin (Table 3.3) for positive identification of the endothelial phenotype. Slides were then washed and incubated with appropriate secondary antibodies (Table 3.3) at room temperature, for 45 minutes, in combination with DAPI (4-6-diamidino-2-phenylindole hydrochloride) (Table 3.3) for assessment of for nuclear integrity. Finally, coverslips were mounted in a glycerol and mowiol 4–88 solution in 0.2 m Tris buffer (pH 8.5) with addition of 0.1% DABCO anti-fade medium.

Table 3.3. Primary and secondary antibodies used for the endothelial cells (EC)s immunofluorescence method.

Primary Antibodies			
Antibody	**Species**	**Isotype**	**REF code, Supplier info.**
VE-cadherin	Mouse	IgG2a	NB600-1409, Novus, UK
	Mouse	IgG2a	Ab7047, Abcam, UK - Discontinued
eNOS	Mouse	IgG1	610297, BD, USA
peNOS Ser^{1177}	Rabbit	IgG	07-428-I, Merck
PECAM-1	Mouse	IgG1	Ab24590, Abcam, UK
PECAM-1 Tyr^{713}	Rabbit	IgG	BS4666, Bioworld Technology, USA
NOX-2	Rabbit	IgG	Gift - Prof Mark Quinn, Montana State University
p53	Mouse	IgG1	Ab26, Abcam, UK
p21	Rabbit	IgG	Ab109520, Abcam, UK
p16	Rabbit	IgG	Abc51243, Abcam, UK
ET-1	Rabbit	IgG	PA3-067, Thermo Fisher Scientific, USA
NF-kB	Mouse	IgG1	NB100-56712, Novus, UK
NT	Mouse	IgG2b	Ab7048, Abcam, UK
Secondary antibodies			
Goat anti-Mouse IgG2a - Alexa Fluor 488			A-21131, Thermo Fisher Scientific, USA
Goat anti-Mouse IgG1 - Alexa Fluor 546			A-21123, Thermo Fisher Scientific, USA
Goat anti-Rabbit IgG - Alexa Fluor 546			A-11035, Thermo Fisher Scientific, USA
Goat anti-Rabbit IgG - Alexa Fluor 633			A-21070, Thermo Fisher Scientific, USA
Goat anti-Mouse IgG2b - Alexa Fluor 546			A-21143, Thermo Fisher Scientific, USA
Goat anti-Mouse IgG1 - Alexa Fluor 633			A-21126, Thermo Fisher Scientific, USA

Vascular Endothelium (VE) – Cadherin; endothelial nitric oxide synthase (eNOS); phospho- endothelial nitric oxide synthase (peNOS Ser^{1177}); platelet endothelial cell adhesion molecule 1 (PECAM-1); phospho-platelet endothelial cell adhesion molecule 1

at tyrosine ^{713} (PECAM-1 Tyr^{713}); NADPHoxidase (NOX)2; tumor protein (p53); cyclin-dependent kinase inhibitor 1 (p21); cyclin-dependent kinase inhibitor 2A (p16); Endothelin (ET)-1; nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB); Nitrotyrosine (NT).

3.5.2. Image capture

Images were acquired using an inverted confocal microscope (Zeiss LSM-710, Carl Zeiss, Germany) with a 63x 1.3NA oil immersion objective. Positive staining for VE-cadherin coupled with a single intact nucleus was used to reliably select ECs [66]. DAPI was excited using the 405 nm line of the diode laser and detected with 371-422 nm emission. Alexa fluor 488 was excited with the 488 nm line of the argon laser and detected with 493–559 nm emission. Alexa Fluor 546 and 633 fluorophores were excited with 543 nm and 633 nm lines of the helium–neon laser and 548–623 nm and 638–747 nm emission, respectively. The images were acquired at a resolution of 1,024 X 1,024 pixels and stored in 24-bit tagged image format file format.

3.5.3. Image analysis

All images were then analysed using ImagePro Plus 5.1 (Media Cybernetics Inc, Bethesda, MD, USA).

To ensure only the cytosolic fraction was assessed nuclear regions of the ECs, identified through the DAPI stain, were extracted from the rest of the ECs image identified using the VE-cadherin image (Figure 3.8). The resulting mask was then overlaid onto the corresponding protein of interest image. Mean fluorescence intensity of the protein of interest signal was then quantified within the endothelial cytosolic specific area. In **Chapter 5,** EC protein expression data are reported as ratios to

human umbilical vein endothelial cells (HUVEC) protein expression. Reporting ratios minimizes the possible confounding effects of different staining sessions. In **Chapter 7** the relative difference between pre and post HE slides was assessed. As pre and post HE slides were stained and imaged in the same batch it was not necessary to present data as a ratio of ECs to HUVEC. A single technician analyzed each batch of slides. Technicians were blinded to subject identity during the staining and analysis procedures.

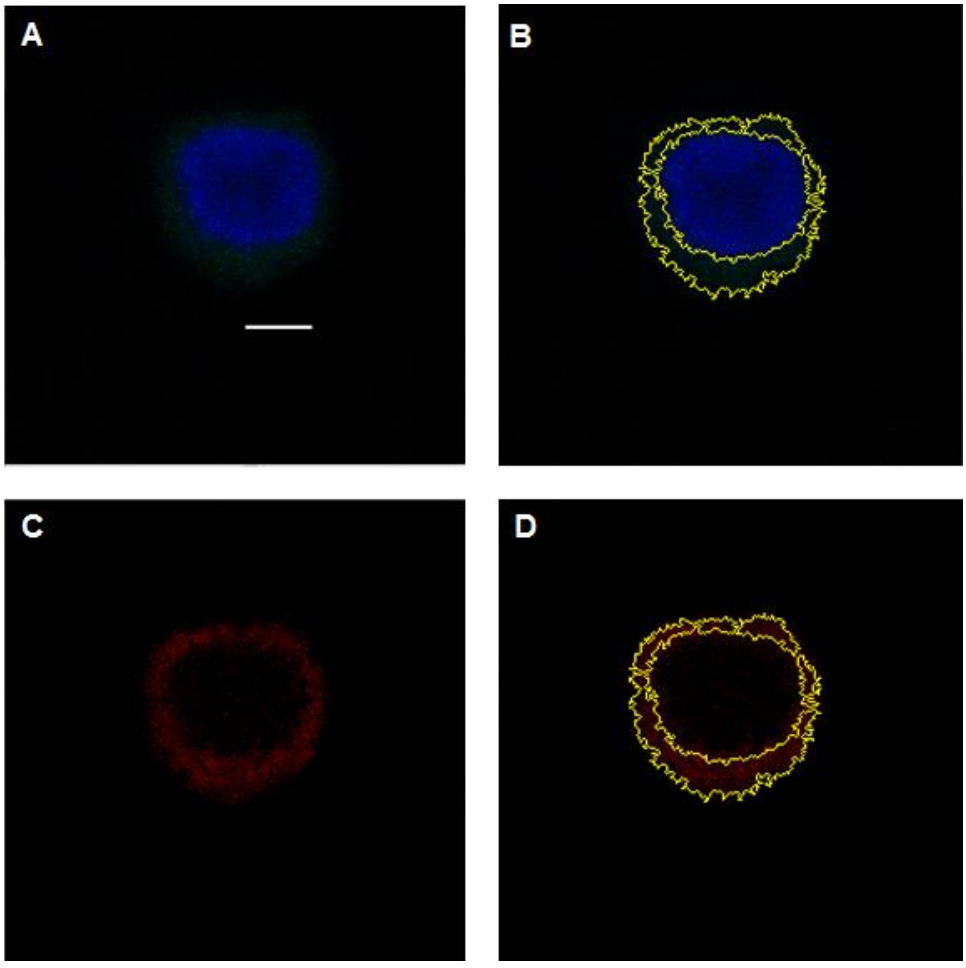


Figure 3.8. Image analysis for quantitative immunofluorescence. Fluorescence microscopy images were captured and processed using Image-Pro Plus 5.1. Following image capture (bar = 2.5 um) (A), an outline of the endothelial cell (EC) was created

using the selection of a standardized signal intensity threshold tool (B). The EC was then transferred to the corresponding protein of interest (C). The staining intensity of the protein of interest was then quantified within the endothelial outline (D).

3.6. Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 25.0 (SPSS Inc., Chicago, IL, USA). Allometric scaling was performed on FMD data to control for differences in baseline diameter [352]. Paired t-test, independent t-test, one-way ANOVA, two-way repeated ANOVA, and a linear mixed model were mainly used to detect differences among variables. Pairwise comparisons were assessed when significance and/or interaction effects were detected. Pearson correlations were used to detect any relations between variables. Details of specific statistical tests used are reported in each Chapter. Data were reported as mean±SD. Statistical significance was recognized when a *P* value <0.05 was observed.

CHAPTER 4 – Exercise-induced vasodilation in the radial artery is not impaired following catheterization damage in coronary artery disease patients.

This Chapter has been published in the American Journal of Applied Physiology.

Tryfonos A, Cocks M, Mills J, Green DJ, and Dawson EA. Exercise-induced vasodilation is not impaired following radial artery catheterization in coronary artery disease patients. *Journal of applied physiology (Bethesda, Md : 1985)* 128: 422-428, 2020.

*Methods section has been edited to avoid repetitions.

4.1. ABSTRACT

Diagnosis and treatment for coronary artery disease (CAD) often involves angiography and/or percutaneous coronary intervention. However, the radial artery catheterization required during both procedures may result in acute artery dysfunction/damage. Whilst exercise-based rehabilitation is recommended for CAD patients following catheterization, it is not known if there is a period when exercise may be detrimental due to localized catheter-induced damage. Animal studies have demonstrated exercise-induced paradoxical vasoconstriction, in damaged arteries, post-catheterization. This study aimed to examine arterial responses to acute exercise following catheterization. Thirty-three CAD patients (65.8±7.3yr, 31.5±6.3kg.m^{-2}, 82%♂) undergoing transradial catheterization were assessed pre- and 1 week post-catheterization. Radial artery (RA) diameter and shear rate were assessed during handgrip exercise (HE), in both the catheterized (CATH) and control (CON) arms. Endothelial function was also assessed via simultaneous bilateral radial flow mediated dilation (FMD) at both time-points. We found that the increase in RA diameter and shear stress in response to HE ($P<0.0001$) was maintained post-catheterization in both the CATH and CON arms, whereas FMD following catheterization was impaired in the CATH [6.5±3.3% to 4.7±3.5% ($P=0.005$)] but not in the CON [6.2±2.6% to 6.4±3.5% ($P=0.797$)] limb. Whilst endothelial dysfunction, assessed by FMD, was apparent 1 week post-catheterization, the ability of the RA to dilate in response to exercise was not impaired. The impact of catheterization and consequent endothelial denudation on vascular dys/function in humans may therefore be stimulus specific and a highly level of redundancy appears to exist that preserves exercise-mediated vasodilator responses.

Key words: acute exercise, arterial function, catheterization-induced damage, coronary artery disease

New & Noteworthy

Despite depressed flow-mediated endothelium-dependent dilation following catheterization-induced damage, radial artery responses to handgrip exercise were preserved. This suggests that arterial responses to catheterization may be stimulus specific and that redundant mechanisms may compensate for vasodilator impairment during exercise. This therefore raises the question about which test is more relevant for examining arterial function and health and their relationship to future risk. It also highlights the difficulty with comparing different assessments of arterial function, more specifically, dilation in response to FMD and an exercise-induced increase in blood flow. Nonetheless, the response to exercise may suggest that patients are safe to begin exercise-based rehabilitation at 1 week following catheterization.

4.2. INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality worldwide [1], with coronary artery disease (CAD) the primary cause of CVD death [21]. Catheterization procedures such as percutaneous transluminal coronary angiography (PTCA) and/or percutaneous coronary intervention (PCI; angioplasty), are routinely used in the diagnosis and treatment of CAD [145, 146, 353]. However, such procedures are likely to mechanically damage ECs [48], leading to artery dysfunction. Indeed, previous studies have reported endothelial dysfunction in both catheterized coronary [150, 354] and peripheral arteries [46, 355] following PTCA and/or PCI.

Whilst exercise training is generally recommended for CAD patients [22], catheterization-induced arterial damage may transiently elevate the risk of cardiac events when the stimulus of exercise is superimposed. Indeed, previous animal studies have demonstrated that endothelial denudation (catheter and balloon inflation utilised to damage/denude the artery) results in ‘paradoxical’ vasoconstriction of damaged arteries in response to exercise [15]. If such responses are apparent in humans, there may be a basis to suggest delaying cardiac rehabilitation, post-catheterization. Although assessment of flow-mediated dilation (FMD) post-catheterization may provide useful information about arterial recovery, and therefore the safest to begin exercise rehabilitation post-catheterization, there is currently no data on the response of human arteries to exercise stimuli following catheterization-induced endothelial damage. Given the complex mechanisms by which exercise regulates blood flow [50], the vascular response of damaged arteries at rest or in response to FMD may be different from the arterial response to exercise. The aim of this study was to examine conduit arterial responses to acute exercise pre- and post-

catheterization in humans. Vascular function was measured in radial arteries, as they are comparable in histopathology and size with coronaries [44], and thus, may represent a useful surrogate of what happening in coronaries post-catheterization. We assessed vascular function by using both flow-mediated dilation (FMD) and handgrip exercise (HE), pre- and post-catheterization. Additional vascular parameters, such as blood velocity, blood flow, shear rate (mean, anterograde and retrograde), as well as blood pressure, handgrip strength and rating of perceived exertion (RPE), were secondary outcomes. We hypothesized that vascular function, assessed via FMD and the response to HE, would be impaired 1 week following PTCA and/or PCI in the catheterized arm, but not in the contralateral control artery.

4.3. METHODS

Participants and Ethical Approval

Thirty-three patients undergoing prospective transradial cardiac catheterization (PTCA and/or PCI) for known or suspected CAD were recruited from Liverpool Heart and Chest Hospital (LHCH). Patients gave written informed consent. Patients were excluded if they had a recent acute coronary syndrome or transradial cardiac catheterization within the last 3 months. This study conformed to the Declaration of Helsinki, and ethical approval was obtained from the Liverpool East NHS Research Ethics Committee (REC 13/NW/0088).

Study design

Vascular function measurements were assessed prior to, and 1 week post-catheterization (PTCA and/or PCI). Both experimental visits were completed in a quiet room, between 0800 and 1100 hrs and patients were fasted (including caffeine and

alcohol) and asked to abstain from exercise and cigarettes for 12 hours before each visit [343]. Diabetic patients had a standardised breakfast (porridge or plain toast), which was the same on both occasions. The pre-assessment was undertaken on the day of the prospective catheterization, before the intervention (~1-4 hours). Experimental visits included two tests (bilateral radial artery FMD and bilateral HE), in this specific order, undertaken in both the catheterized (CATH) and the contralateral (CON) arm. Due to limited available time to test in the hospital setting (patients needed to be prepared for catheterization), we decided to use a standard protocol of testing with no randomisation. To randomise the order and perform handgrip exercise first in half patients, we would have had to add a resting period in the supine position before the FMD, which would have increased the length of the testing, and been a greater burden on the patients.

Transradial Cardiac Catheterization

PTCA and/or PCI was performed predominantly via the right radial artery (RA) (9% via left radial artery), as descripted in **Chapter 3**.

Experimental procedures

Maximal voluntary contraction (MVC) of both arms was measured, during both visits, using a dynamometer (Takei 5420 Grip-D Digital Hand Grip Dynamometer, Japan), as descripted in **Chapter 3**. Patients then rested in the supine position for >10 minutes to ensure that all hemodynamic variables stabilised. Blood pressure (BP) and heart rate (HR) were measured using an automated sphygmomanometer (GE Pro 300V2, Dinamap, Tampa, FL, USA), after the resting period. Two 12-MHz multi-frequency linear array probes, attached to two high-resolution ultrasound machines (T3000 or

Terason u-smart 3300; Teratech, Burlington, MA, USA) were used to image the RA (10-15 cm proximal from the scaphoid bone in the wrist), for both tests (see **Chapter 3** for more details). The same ultrasounds and sonographers were used between the visits, and within participants.

Bilateral radial artery FMD

FMD was measured in both radial arteries, as descripted in **Chapter 3**.

Bilateral HE

Following the FMD test, patients performed an incremental handgrip exercise (HE) protocol, while in the seated position (see **Chapter 3** for details). Participants completed 3 minutes of HE at each of 5, 10 and 15% pre-determined MVC, with 1-minute rest between these bouts. Diameter and velocity recordings were obtained from the RA, before the HE, and three times during the 1-minute rest at the end of each HE intensity (at 5% MVC, 10% MVC and 15% MVC). Rating of perceived exertion (RPE) on a 1-10 scale (1: no effort to 10: maximal effort) was taken at the end of each HE bout.

Data analysis

Custom-designed edge-detection and wall-tracking software was used to analyse both the FMD and HE, in order to minimise the investigator bias [342, 348]. See **Chapter 3** for more details. For HE analysis in this Chapter, changes in diameter, velocity, blood flow AUC (mean, anterograde, and retrograde), and SRAUC (mean, anterograde and retrograde) were calculated as averages (usually a 1-minute recording), before, and

during the 1-minute rest between the incremental HE bouts. For further analysis of HE parameters, baseline values taken before HE (Pre-Ex) and the peak value achieved (Peak-Ex) during HE (either at 5%, 10% or 15% MVC) were compared.

Statistics

All analyses were performed using IBM SPSS statistics for Windows, version 25.0 (Armonk, NY: IBM Corp.). For FMD, allometric scaling was performed to control for differences in baseline diameter [352] and then a mixed-linear model (arm*time), controlling for baseline diameter, was undertaken. For HE, a mixed-linear model (arm*time*exercise) was used. A mixed-linear model was also used to analyze the differences in MVC, and RPE, between arms and/or pre-post catheterization. Paired *t*-test were used to assess BP and HR changes pre- to post-catheterization. Pairwise comparisons were performed, using the Fisher's least significant difference (LSD), when significant main or interaction effects were detected. Data are presented as mean±SD and alpha significance was set at $P \leq 0.05$.

4.4. RESULTS

Patient characteristics and medications, prior to catheterization, are shown in Table 4.1. The majority of the patients were taking at least one of the following: aspirin, beta-blocker, angiotensin-converting enzyme (ACE) inhibitor or angiotensin II receptor blocker (ARB), nitrate or a statin. All 33 patients had successful transradial catheterization; 20 patients had PTCA and 13 patients PCI (1 x no stent, 9 x 1 stent, 2 x 2 stents, 1 x 3 stents). Four patients were referred for coronary artery bypass graft

(CABG) following the diagnostic PTCA. These patients were stable, and their CABG was scheduled more than 1 week following diagnostic PTCA, therefore patients were allowed to attend the follow-up visit 1 week post-catheterization. FMD was performed on all 33 patients, while 29 patients completed the HE protocol (2x equipment failure, 1x avoid exercise due to dizziness following FMD, 1x previous injury to their hand). Arterial patency was not recorded immediately after catheterization, but none of the 33 patients we assessed 1 week post-catheterization using Doppler ultrasound had any apparent radial occlusion.

Impact of catheterization on HE response

There was a main effect of HE on RA diameter, with RA diameter increasing in response to HE (main effect of exercise, $P<0.001$). This exercise-induced vasodilation was similar in both arms and remained unchanged pre- and post-catheterization (time*arm*exercise interaction ($P=0.725$). A significant finding (time*arm interaction, $P<0.001$) suggested that the diameter of the catheterized RA was higher 1 week post-catheterization, compared with pre-catheterization ($P<0.001$), whereas RA diameter was unchanged in the control RA ($P=0.086$) (Table 4.2). There was no difference in percentage change in RA diameter in response to HE, pre- vs post-catheterization, in either arm (Figure 4.1A, B).

There was a significant increase in mean, anterograde and retrograde shear rate in response to HE ($P<0.001$), but there were no differences in these responses between arms ($P=0.138$, $P=0.098$, and $P=0.133$ respectively), or pre- to post-catheterization ($P=0.121$, $P=0.148$, and $P=0.172$ respectively) (Figure 4.2C and Table 4.3). Similarly,

blood velocity, total mean blood flow, anterograde blood flow and retrograde blood flow followed the same pattern, with significant increases in response to exercise ($P<0.001$), but no differences pre- to post-catheterization ($P=0.274$, $P=0.275$, $P=0.286$ and $P=0.614$ respectively) or between arms ($P=0.102$, $P=0.157$, $P=0.107$ and $P=0.064$ respectively) (Table 4.3).

Table 4.1. Characteristics of the study population (n=33).

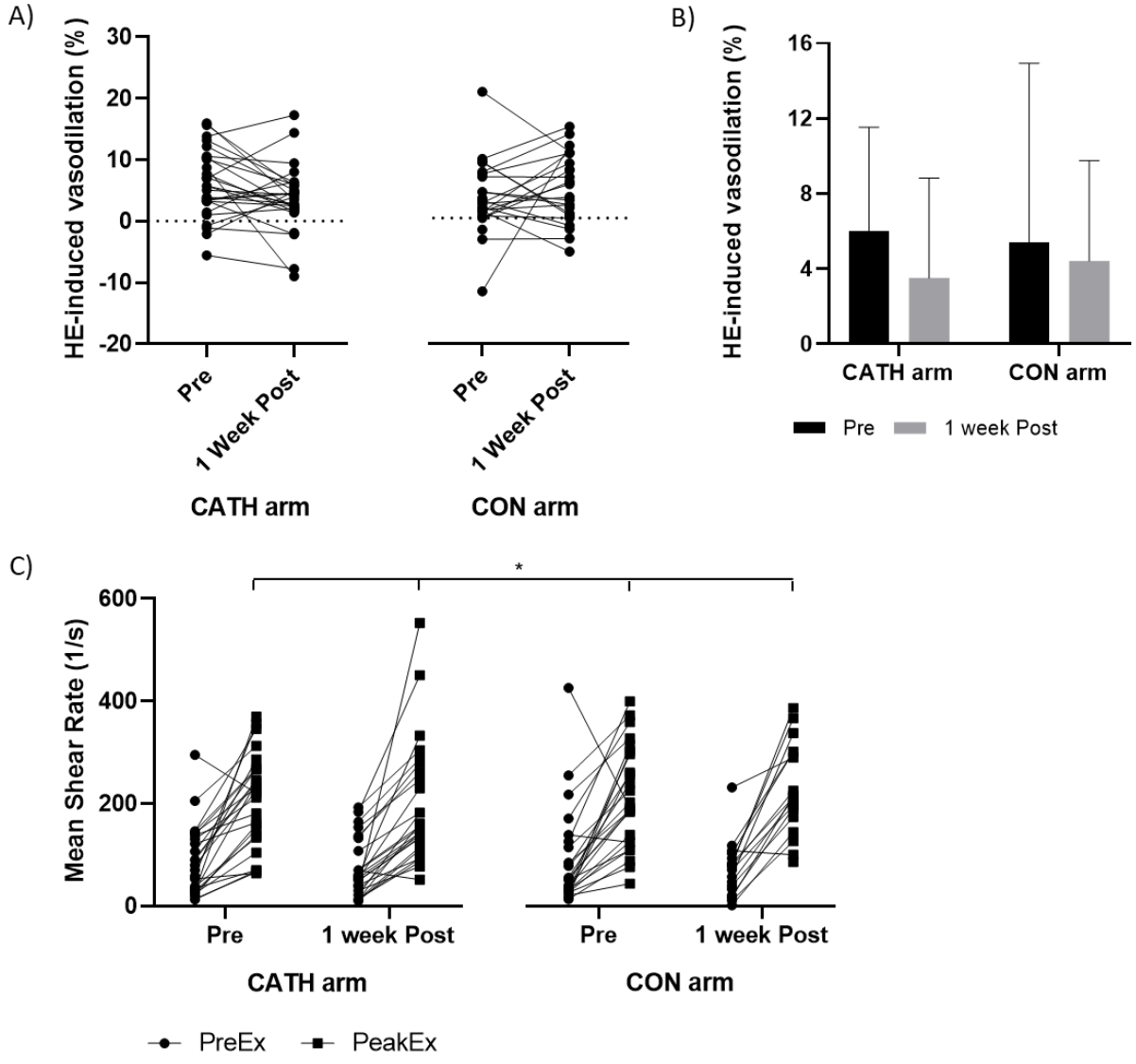
Clinical Characteristic		Mean ± SD or n (%)
Age (years)		65.8 ± 7.3
Sex (males)		27 (81.8)
BMI (m/kg^2)		31.5 ± 6.3
Risk	Diabetes	9 (27.2)
Factors	Hypertension	20 (60.6)
	Hypercholesterolemia	24 (72.5)
	Current smoker	3 (9.1)
	Ex-smoker	17 (51.5)
	Positive family history	20 (60.6)
Previous transradial catheterization (PTCA and/or PCI)		20 (60.6)
Previous CABG		0 (0)
Previous MI > 3 months		13 (39.4)
Medications	Aspirin	29 (87.8)
	Clopidogrel	2 (6)
	Beta-Blocker	20 (60.6)
	ACEI/ARB	22 (66.7)
	Nitrate	23 (69.7)
	Statin	26 (78.8)
	Calcium-Blocker	9 (27.3)
	Diuretics	7 (21.2)

BMI: body mass index; PTCA: percutaneous transluminal coronary angiography; PCI: percutaneous coronary intervention; CABG: coronary artery bypass graft; MI: myocardial infraction; ACEI: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker.

Table 4.2. Vascular responses to handgrip exercise (HE). Vascular parameters, at rest, prior HE (PreEx) and the peak value to HE (PeakEx), in the catheterized radial artery (CATH) and the contralateral radial artery (CON), before the catheterization (Pre) and at 1 week post-catheterization.

	CATH arm		CON arm	
Diameter (mm)	**Pre**	**1 week Post**	**Pre**	**1 week Post**
PreEx	2.7±0.5	2.9±0.4	2.8±0.5	2.7±0.5
PeakEx	2.9±0.5*	3.0±0.4*	2.9±0.6*	2.8±0.5*
Velocity (cm/s)				
PreEx	6.1±4.0	5.0±3.9	6.8±6.0	6.0±4.4
PeakEx	15.0±6.7*	13.9±7.7*	16.5±10.3*	15.8±8.8*
Total Blood Flow (ml/min)				
PreEx	20.5±15.2	20.2±15.8	25.0±28.0	21.7±22.0
PeakEx	54.7±25.4*	56.1±28.4*	67.7±46.6*	56.0±33.7*
Anterograde Blood Flow (ml/min)				
PreEx	23.5±14.9	22.7±15.3	28.5±27.1	25.7±21.1
PeakEx	54.9±24.6*	56.4±28.1*	68.1±46.2*	56.4±33.5*
Retrograde Blood Flow (ml/min)				
PreEx	-3.0±2.6	-2.4±1.8	-3.5±3.4	-4.0±5.5
PeakEx	-1.2±1.7*	-1.2±1.4*	-1.6±2.2*	-1.0±1.3*
Anterograde Shear rate (1/s)				
PreEx	108.1±67.3	79.5±56.5	115.0±91.1	104.7±58.4
PeakEx	227.4±122.8*	194.9±118.9*	238.2±171.0*	232.4±130.4*
Retrograde Shear Rate (1/s)				
PreEx	-13.2±13.2	-8.7±6.9	-13.3±13.8	-15.1.2±20.5
PeakEx	-4.8±7.2*	-3.5±3.6*	-7.1±13.1*	-3.7±5.7*

Results are presented as mean±SD, n=29 (24 males). A mix-linear model (arm*time*exercise) with Fisher's least significant difference post hoc for pairwise comparisons was used. *Significantly different from PreEx, main effect of exercise (P<0.05)



<u>Figure 4.1.</u> Vascular responses to handgrip exercise (HE).

Percentage change in RA diameter following HE, individual data (A) and summary data, presented as mean±SD n=29 (24 males), in the catheterized RA (CATH arm)

and contralateral control RA (CON arm), pre- and 1 week post-catheterization. Mean shear rate, individual data (C), prior to exercise (PreEx) and at peak (PeakEx), in the CATH arm and CON arm, pre- and 1 week post-catheterization. *Significantly different from PreEx, main effect of exercise intensity (P<0.05).

Impact of catheterization on FMD

There was a significant impact of catheterization on FMD (time*arm interaction), when controlling for baseline diameter (P=0.034), and without baseline diameter normalization (P=0.011). There was a significant reduction in FMD in the catheterized RA [6.5±3.3% to 4.7±3.5% (P=0.005)], with no change in the non-catheterized RA [6.2±2.6% to 6.4±3.5% (P=0.797)] following catheterization (Figure 4.2).

As with the HE data, baseline artery diameter during the FMD test significantly differed after catheterization (significant time*arm interaction for P=0.046). When pairwise comparisons were performed, an increase in baseline diameter 1 week post-catheterization was observed in the catheterized RA, compared to pre-catheterization (P=0.009). There was no change in the control RA (P=0.785). Baseline RA diameter was not different between arms before the catheterization (P=0.707) but was significantly higher in the catheterized RA compared to the control arm 1 week post-catheterization (P=0.016). There was no change in peak diameter, time to peak diameter or shear rate under the curve (SRAUC) (Table 4.3).

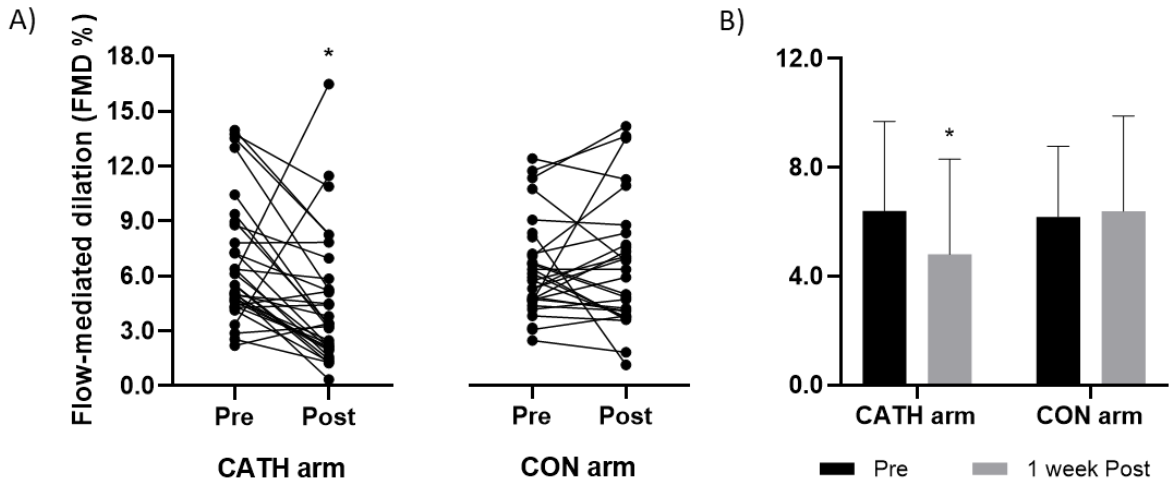


Figure 4.2. Changes in flow-mediated dilation (FMD %) in the catheterized radial artery (CATH) and contralateral arm (CON), pre- and 1 week post-catheterization.

A) Individual data, B) Summary data, presented as mean±SD n=33 (27 males). A mix-linear model (arm*time) with Fisher's least significant difference post hoc for pairwise comparisons was used. *Significantly different from Pre, main interaction effect of time*arm (P<0.05).

Table 4.3. Baseline diameter, peak diameter, time to peak and SRAUC associated with the FMD tests before the procedure (Pre) and at 1 week post-catheterization, in the catheterized (CATH) arm and the contralateral (CON) arm.

	CATH arm		CON arm	
	Pre	**1 week Post**	**Pre**	**1 week Post**
Baseline diameter (mm)	2.82±0.66	3.04±0.53*	2.85±0.53	2.73±0.53†
Peak Diameter (mm)	3.00±0.67	3.18±0.52	3.03±0.54	3.01±0.58
Time to peak (s)	50.8±25.1	56.7±27.9	66.0±32.4	57.4±34.2
SRAUC (s^{-1} 10^3)	18.5±12.4	15.0±8.8	16.3±10.6	14.0±9.3

SRAUC: shear rate area under the curve. Results are presented as mean±SD, n=33 (27 males). A mix-linear model (arm*time) with Fisher's least significant difference post hoc for pairwise comparisons was used. *Significantly different from Pre (P<0.05), †Significantly from CATH arm (P<0.05).

Impact of catheterization on systemic haemodynamic measurements, MVC, and RPE

There was no change in BP or HR pre- to post-catheterization (Table 4.4). MVC was higher in the catheterized arm compared with the control arm (P=0.024), during both visits. MVC was unchanged following catheterization (P=0.265; Table 4.4). RPE was increased with incremental HE (main effect P<0.001), but there was no effect of catheterization (P=0.588). When pairwise comparisons were examined, RPE at 5% MVC was lower than 10% MVC (P=0.001) and 15% MVC (P<0.001), but there was no difference between the RPE at 10% and 15% MVC (P=0.177) (Table 4.4).

Table 4.4. Resting haemodynamic measurements, MVC in the catheterized arm (CATH) and control arm (CON), and RPE as reported during HE, pre and 1 week post-catheterization.

	Pre	1 week Post	P value
Haemodynamic measurements			
SBP (mmHg)	138±19	133±23	0.151
DBP (mmHg)	81±10	78±10	0.121
MAP (mmHg)	100±11	94±21	0.080
HR (beats per min)	62±12	61±11	0.428
MVC (Kg)			
CATH arm	32.6±9.7	33.7±9.7	0.297
CON arm	31.2±9.5	31.8±7.9	0.592
RPE (1-10) during incremental HE			
5% MVC	2.1±1.5†,‡	1.7±1†,‡	0.329
10% MVC	3.8±2.0*	3.5±1.9*	0.562
15% MVC	5.4±2.2*	5.7±2.1*	0.109

SBP: systolic blood pressure; DPB: diastolic blood pressure; MAP: mean blood pressure; HR: heart rate; MVC: maximal voluntary contraction; RPE: rate of perceived excursion (1: no effort to 10: maximal effort). Results are presented as mean±SD, n=33 (27 males). A paired t-test was used to assess BP and HR. A mixed-linear model was used to analyze MVC and RPE, between arms and/or pre-post catheterization with Fisher's least significant difference post hoc for pairwise comparisons (P<0.05). *Significantly different from 5% MVC, †Significantly different from 10% MVC, ‡Significantly different from 15% MVC.

4.5. DISCUSSION

This study aimed to examine the impact of catheterization on radial artery function in CAD patients. We assessed two vascular responses: a) a shear stress mediated assessment of endothelium-dependent dilation (FMD), which is largely mediated by nitric-oxide, and b) the response to handgrip exercise (HE) which represents a mechanistically complex but ecologically valid measure of vascular function. To our knowledge, this is the first study in humans to examine radial artery responses to exercise following catheterization. In contrast to the hypothesis, we observed that vasodilator responses to exercise were relatively preserved 1 week following catheterization, whereas in support of the hypothesis, there was evidence for impairment in FMD responses post-catheterization. These data suggest that the impact of catheterization on functional arterial responses may be stimulus specific.

Our observation that RA dilation in response to exercise was not impaired 1 week post-catheterization contrasts with some previous studies in animals, which have reported a paradoxical vasoconstriction in response to exercise up to 6 days following endothelial denudation [15, 16]. Although we do not know for certain whether impaired exercise-induced dilation may be apparent earlier days post-injury, we have chosen to evaluate function at 1 week following catheterization as we believe that it is feasible that exercise-based cardiac rehabilitation can start around this time, but is unlikely to start sooner. In addition, two studies in patients performing supine bicycle exercise during a follow-up PTCA reported an exercise-induced constriction in the treated vessels, at 6 months post-PCI with 1st generation [330] and at 16 months with 2nd generation drug-eluting stents (DES) [331]. However, this impairment may indicate the presence of long-term complications of stenting, such as in-stent restenosis [356],

rather than the effects of catheterization-induced damage *per se*. In addition, there were no baseline assessments in either study and it is therefore possible that impairment was the result of advanced atherosclerotic disease [326] apparent prior to catheterization. In any event, the paradoxical constriction of catheterized arteries in response to exercise reported in these studies may contribute to exercise-induced myocardial ischemia post-catheterization [15]. This may be mediated by the impact of noradrenaline (NE) and adrenaline (E) or other vasoconstrictors released during exercise, which may impact directly on the vascular smooth muscles or via the endothelium [357]. Indeed, endothelial damage following catheterization has been proposed as a factor to take into account when considering the optimal time to begin exercise rehabilitation [13].

In the present study, we assessed the short-term impact of catheterization on arterial responses to exercise, by evaluating the responses of the RA before and 1 week post-procedure, alongside a contralateral internal control. This experimental design suggests that our result, indicating preserved arterial response to exercise, is robust. Radial arteries are comparable in size and histopathology to coronaries [44]. Whilst our results cannot be directly extrapolated to other arterial beds, they nonetheless suggest that conduit arteries can retain their ability to dilate in response to exercise following catheterization. This may have implications for recommendations pertaining to safe timing of the uptake of cardiac rehabilitation. Two large-scale studies conducted to determine the incidence of cardiac events induced by exercise in patients who underwent PCI, concluded that performing submaximal or maximal exercise 1 to 14 days post-PCI is safe [302, 303]. Although such events may be trigged by number or reasons, including arrhythmia which are not related to vascular function, the fact

that performing exercise a few days post catheterization is not related to adverse events and that exercise-induced dilation is preserved remains an important finding.

Our exercise outcomes are informed by the fact that we also collected FMD data, relating to endothelial function. In contrast to the exercise-mediated dilation, FMD was impaired in the catheterized arm 1 week post-catheterization. There was no change in the contralateral arm, suggesting that the impact of catheterization was localised and not systemic. Our FMD findings are consistent with previous studies in humans, which have indicated an immediate (within 24 hours) reduction in FMD in the catheterized artery, but not in the contralateral artery, following transradial catheterization [45-47, 49, 163, 355]. Although a recent study observed impaired endothelial function 1 week post-procedure (lower FMD in the catheterized arm compared to the control arm) [48], this study did not measure change in function pre- to post-procedure. Consequently, our FMD findings are the first to report direct data on local endothelial impairment 1 week following PTCA and/or PCI. It has been proposed that FMD evaluates endothelium-dependent dilation, which is largely nitric oxide (NO)-mediated [346]. It is therefore likely that PTCA and/or PCI impair NO production in the catheterized vessels. Reduced NO production has been associated with proliferation and migration of VSMC, as well as the activation of platelet cascades, increasing the risk for restenosis and thrombosis [17]. However, it is worth noting that the present study does not provide information about the degree of endothelial damage induced by catheterization. Previous data suggests injury to the radial artery in approximately 1/5th of patients following transradial catheterization, 1/3rd of which have significant dissection extending into media layer [48]. Therefore, we do not know whether this FMD impairment is related completely to endothelial damage or also includes damage

to the VSMC. Interestingly, some observations indicate impaired FMD in non-catheterized vessels following PCI [175, 189, 191], suggesting that the endothelial dysfunction induced by catheterization may also reflect systemic arterial dysfunction, potentially due to elevated oxidative stress, inflammation and platelet aggregation induced by invasive procedures. Importantly, here we have shown that effects remained localized to the catheterized vessels.

Regulation of blood flow during exercise is complex, involving a number of mechanisms and vasoactive compounds, with multiple interactions and redundancy [358, 359]. Our finding that catheterization impaired FMD, but not HE responses, suggests that vascular responses to exercise are preserved by redundant pathways other than those purely related to NO-mediated function. In support of this notion, Padilla *et al.* 2006 [360] demonstrated impaired FMD, but preserved responses to handgrip exercise, in healthy subjects following a high-fat meal. Our findings regarding stimulus specific vascular changes highlight the importance of applying multiple techniques to evaluate arterial function. Indeed, previous experiments have indicated that different periods of cuff inflation induce arterial dilation via distinct pathways in humans [346]. Routinely assessing vascular responses to exercise could provide an ecologically valid assessment to complement FMD measures in future studies, particularly as it is the most relevant test to provide insights regarding exercise-based rehabilitation in CAD patients following catheterization.

Previous studies of the brachial artery have indicated that, as is the case for FMD responses, HE mediated arterial dilation is shear stress mediated [309, 361, 362]. For

example, McPhee and Pyke (2018) [362] suggested that handgrip exercise resulted in similar vasodilation induced by reactive hyperaemia (FMD) and sustained shear (HE). In contrast, there are other studies suggesting that vasodilation in response to reactive and active hyperaemia may be driven by distinct mechanisms [360]. If it can be assumed that HE-mediated dilation of the *radial* artery is shear stress mediated, then our finding that catheterization does not impact HE responses, despite impacting radial FMD, would suggest that the stimulus specificity relates to the nature of the shear stress stimulus. Our approach utilising post-catheterization responses may provide future insight into the sensitivity of different arteries to stimuli that induce distinct shear stress profiles.

In addition to functional changes, we have also demonstrated that structural changes may occur after catheterization. There was an increase in RA diameter in the catheterized arm, but not in the contralateral arm, 1 week post-catheterization. As expected, this was observed prior to both FMD and HE. Previous studies have reported similar findings of elevated RA diameter in the catheterized arm 24 hours post-catheterization [45, 46, 49, 163]. Collectively, our findings suggest that such structural modifications remained apparent 1 week post-catheterization and therefore should be consider as a consequence of catheterization and not just an immediate reaction of the artery to the procedure. The time-course of structural adaptation or remodelling following catheterization is an important question that should be addressed in future studies.

This study had a number of limitations. We did not control for age, gender, pre-existing disease (diabetes, hypertension, peripheral artery disease), history of smoking or medication use (including potential changes pre- to post-intervention). However, our patient population are typical of those attending for interventions and our repeated measures study design and contralateral within-subjects control artery somewhat mitigates these limitations. In addition, we were not able to control for different introducer catheter size, or compression time, which were both clinically determined, as indicated. These may have affected arterial patency and possibly vascular outcomes. In our study, 6F introducers were mostly used (28 out of 33 patients), with 5F and 7F introducers used in 4 and 1 patient, respectively. However, we performed a supplementary mix-model liner regression, for FMD and HE responses, with catheter size as a covariate and this did not affect the study outcomes or interpretation. Analysis was not blinded between pre- and post-catheterization trials, but researchers were blinded to artery condition (catheterized or non-catheterized). To overcome this limitation, analysis was double checked by two observers who were looking for the best curve (FMD) and best-tracking diameter/velocity for HE data. With respect to exercise responses, we have analysed the 1-min rest period between HE intensities, which may result in underestimation of peak exercise responses due to potential gradual loss of hyperaemia over time. However, HE involved muscle contraction which significantly affected the quality of images (lower average diameter during 1 min exercise due to muscle contractions). To support the above, we have conducted pilot work in healthy males (Chapter 3) where we confirmed that these HE intensities were adequate to dilate the radial artery during the 1-min rest. Finally, the contralateral within-subjects control artery somewhat mitigates these methodological limitations.

In conclusion, this study provides important information regarding arterial function following catheterization in humans. Our data showed that, although catheterization induced localised impairment in flow mediated dilation, the ability of the RA to dilate in response to exercise following catheterization-induced damage was largely unaffected. This highlights that vascular responses to catheterization may be stimulus specific. Since arterial responses to exercise were relatively preserved following catheterization, it may be safe to undertake exercise-based rehabilitation soon after catheterization procedures, although this should be confirmed in other cohorts and in larger samples.

CHAPTER 5 - Association between atherogenic risk-modulating proteins and endothelium dependent flow-mediated dilation in coronary artery disease.

5.1. ABSTRACT

Endothelial dysfunction is an early and integral event in the development of atherosclerosis and coronary artery disease (CAD). Reduced NO bioavailability, elevated oxidative stress, vasoconstriction, inflammation and senescence are all implicated in endothelial dysfunction. However, there is limited evidence regarding associations between these pathways and direct *in vivo* bioassay measures of endothelial function in patients with diagnosed CAD. In addition, it is unclear whether endothelial function and the expression of such atherogenic pathways vary across the different stages of CAD. This study aimed: a) to examine the relationships between *in vivo* measures of vascular function and the expression of atherogenic risk-modulating proteins in endothelial cells (EC)s isolated from the radial artery of CAD patients and b) to compare endothelial function and the expression of such pathways between CAD patients with different clinical outcomes (obstructive vs non-obstructive coronaries). Fifty-six patients underwent transradial catheterization. Upon diagnosis patients were divided into two groups, non-obstructive CAD (n=18) and obstructive CAD (n=38). Prior to catheterization radial artery vascular function was assessed using a) flow-mediated dilation (FMD), and b) diameter change as function of handgrip exercise intensity (5, 10 and 15% of MVC). Freshly isolated ECs were obtained from the radial artery during transradial catheterization and protein content of eNOS, NAD(P)Hox subunit 2, NFκB, ET-1 and the senescence markers p53, p21 and p16 were evaluated alongside nitrotyrosine abundance and eNOS Ser^{1177} phosphorylation. FMD was positively associated with eNOS Ser^{1177} phosphorylation (r=0.290, P=0.037), and protein content of p21 (r=0.307, P=0.027) and p16 (r=0.426, P=0.002). No associations were found between FMD and markers of oxidative stress, vasoconstriction or inflammation. HE% was not associated with any of the EC proteins

or FMD. Neither FMD, diameter change during handgrip exercise or endothelial expression of atherogenic risk-modulating proteins were different between patients with obstructive and non-obstructive CAD. A number of associations were observed between expression of atherogenic risk-modulating proteins. The main associations observed in this study provide a novel insight on the molecular mechanisms related to vascular function in CAD.

5.2. INTRODUCTION

Cardiovascular disease (CVD) is the number one cause of global mortality, representing 31% of all deaths [1]. Coronary artery disease (CAD) is the leading cause of CVD deaths [21]. Atherosclerosis, a systemic inflammatory process resulting in thickening of arteries, plaque formation and sometimes rupture [2], is the underlying cause of CAD. Endothelial dysfunction has emerged as an early and integral event in the atherogenic process [55]. Indeed, reduced endothelium-dependent flow-mediated dilation of the brachial artery is indicative of largely nitric oxide (NO)-mediated endothelial dysfunction and has been associated with cardiovascular events [10].

Endothelial dysfunction is often characterised by a reduction in nitric oxide (NO) bioavailability. Endothelial nitric oxide synthase (eNOS) is the key enzyme for endothelial NO production [7]. eNOS protein content and serine^{1177} (Ser^{1177}) phosphorylation together determine eNOS activity and NO production [363]. Reduced NO bioavailability arises not only from impaired NO synthesis, but also reflects NO scavenging by superoxide anions and related reactive oxygen species. A major source of superoxide anion production and NO scavenging in the vascular wall is NAD(P)Hoxidase [91].

Increased superoxide anion production, together with decreased NO synthesis, stimulate expression of endothelin-1 (ET-1), an important vasoconstrictor associated with endothelial dysfunction [76]. Similarly, ET-1 promotes the production of superoxide anions, via NAD(P)Hoxidase activation, and inhibits NO synthesis, creating a vicious cycle between oxidative stress, reduced NO bioavailability and ET-1 expression [364]. Simultaneously, elevated oxidative stress enhances the activation

of nuclear factor κB (NFκB) [108, 109, 117] and senescence pathways [108, 126, 353, 365], which further promote the expression of pro-oxidant and pro-inflammatory markers.

Reduced endothelial NO bioavailability, in combination with elevated oxidative stress [87-89], vasoconstriction [76], inflammation [77] and senescence [126], have all been shown to contribute to the development of endothelial dysfunction and, are therefore considered contributory mechanisms underlying the pathophysiology of CAD. However, there is limited direct evidence of the existence of an association between the activity of these pathways and *in vivo* measurements of endothelial function in patients with diagnosed CAD. Therefore, the primary aims of this study were: a) to determine whether the expression of atherogenic-modulating proteins can predict endothelial function, assessed through flow-mediated dilation (FMD) an *in vivo* bioassay on NO-mediated vasodilator function, in patients with CAD, and b) to investigate if these atherogenic-modulating proteins can also predict the vasodilatory response to exercise in these patients. Hyperaemic responses to exercise represent an integrated response involving autonomic activity, and numerous endothelium- and non-endothelium-dependent vasoactive systems [50]. As such, the vascular response to exercise provides important additional information, when presented alongside measurement of FMD [366]. This study has also a number of secondary aims: a) to compare the *in vivo* measurements of vascular function (FMD, diameter change to exercise) and the expression of atherogenic-modulating proteins between CAD patients with obstructive and non-obstructive coronaries, b) examine whether FMD could predict the arterial response to exercise in these patients, and c) to explore associations between the expression of EC proteins related to endothelial dys/function

in CAD patients. Here we hypothesized that patients with obstructive coronaries would experience lower FMD and arterial response to exercise, and higher expression of atherogenic proteins. In addition, FMD would predict the arterial response to exercise. Finally, oxidative and inflammatory markers would be positively associated with senescence and all would be negatively correlated with eNOS content and phosphorylation at Ser^{1177}.

5.3. METHODS

Participants

Sixty-four patients undergoing prospective percutaneous transluminal coronary angiography (PTCA) and/or percutaneous coronary intervention (PCI; angioplasty) were recruited from the Liverpool Heart and Chest Hospital (LHCH). Patients were excluded if they were unable to give informed consent or had undergone a trans-radial cardiac catheterization or acute coronary syndrome within the last 3 months. Eight patients had angiographically normal coronaries following PTCA and thus, were retrospectively excluded. As such, data from 56 CAD patients were included in this study. A summary of patient characteristics, medications, hemodynamic variables and previous catheterization is included in Table 5.1. Based on angiographic outcomes, patients were allocated to two groups: a) non-obstructive CAD (non-obCAD) (>30<70% stenosis) (n=18) and b) obstructive CAD (obCAD) (>70% stenosis) (n=38). The study conformed to the Declaration of Helsinki, and ethical approval was obtained from the Liverpool East NHS Research Ethics Committee (REC 13/NW/0088).

Table 5.1. Characteristics of the study population (n=56).

Clinical Characteristic		Mean ± SD or n (%)
Age (years)		66.5 ± 8.6
Sex (males)		47 (83.9)
BMI ($kg.m^{-2}$)		29.6 ± 5.3
Mean Blood Pressure (mmHg)		99.5 ± 11.2
Heart rate (beats/min)		61.3 ± 10.1
Previous transradial catheterization (PTCA and/or PCI)		23 (41.1)
Previous CABG		5 (8.9)
Previous MI > 3 months		19 (33.9)
Risk Factors	Diabetes	17 (30.4)
	Hypertension	34 (60.7)
	Hypercholesterolemia	42 (75.0)
	Current smoker	6 (10.7)
	Ex-smoker	34 (60.7)
	Positive family history	39 (69.6)
Medications	Aspirin	50 (89.3)
	Clopidogrel	6 (10.7)
	Beta-Blocker	39 (69.6)
	ACEI/ARB	31 (55.4)
	Nitrate	44 (78.6)
	Statin	46 (82.1)
	Calcium-Blocker	17 (30.4)
	Diuretics	8 (14.3)

BMI: body mass index; PTCA: percutaneous transluminal coronary angiography; PCI: percutaneous coronary intervention; CABG: coronary artery bypass graft; MI: myocardial infarction; ACEI: angiotensin-converting enzyme inhibitor; ARB: angiotensin receptor blocker.

Study design

Patients abstained from exercise, alcohol, caffeine, and cigarettes for 12 hours before attending LHCH for their PTCA and/or PCI [343]. Patients continued their medications on the day of procedure, as instructed by their consultant. Upon arrival, vascular function was assessed in the catheterized radial artery, using two non-invasive measurements: a) flow-mediated dilation (FMD) and b) vascular dilation to incremental handgrip exercise. A standardized period of 10 minutes was observed between these tests to allow artery function to recover to baseline levels. Patients then underwent PTCA and/or PCI, ECs were collected from the catheterized radial artery, as descripted below.

Assessment of vascular function

Patients rested in the supine position for >10 minutes before blood pressure (BP) and heart rate (HR) were measured using an automated sphygmomanometer (GE Pro 300V2, Dinamap, Tampa, FL, USA). For both FMD and handgrip exercise, a 12-MHz multi-frequency linear array probe, attached to a high-resolution ultrasound machine (T3000; Terason, Burlington, MA, USA) was used to image the radial artery (RA) in the catheterized arm, proximal to the wrist (10-15 cm proximal from the scaphoid bone in the wrist).

Flow-mediated dilation (FMD)

This technique has been descripted in **Chapter 3.**

Radial artery response to handgrip exercise

Patients performed a 3 x 3-minute bout of handgrip exercise, in a seated position, at 5%, 10% and 15% of their pre-determined maximal voluntary contraction (MVC) (Takei 5420 Grip-D Digital Hand Grip Dynamometer, Japan), with 1-minute rest between bouts, as descripted in **Chapter 3**. Arterial diameter and velocity recording were measured at baseline and at the end of each exercise bout, during the 1-minute rest.

Data analysis

Custom-designed edge-detection and wall-tracking software was used to analyse both the FMD and handgrip exercise, in order to minimise investigator bias [342, 348]. See **Chapter 3** for more details. With respect to handgrip exercise, changes in diameter, velocity, blood flow area under the curve (AUC) (mean, anterograde, and retrograde), and shear rate area under the curve (SRAUC, mean, anterograde and retrograde) were calculated as averages of ~1 minute recordings, before, and during the 1-minute rest periods between exercise bouts. Importantly for this Chapter, the percentage difference in diameter from the baseline to peak during handgrip exercise (the maximum value at either 5%, 10% or 15% MVC) was calculated (HE%), and used to examine any correlations with other vascular outcomes (FMD, protein content in ECs).

Transradial catheterization and EC collection

PTCA and/or PCI was performed predominantly via the right radial artery (5.8% via left radial artery), as descripted in **Chapter 3.**

All patients received a weight-adjusted dose of heparin. Thirty patients received an additional ~1.5 mg/ml of vasodilator isosorbide dinitrate introduced into the central circulation during the procedure, as indicated by the interventional cardiologist.

Immunofluorescence Microscopy

Details of the procedures used have been descripted in **Chapter 3**. Briefly, cells were recovered by centrifugation. Collected cells were fixed with 3.7% formaldehyde and plated onto glass slides and then frozen at -80°C until analysis. See **Chapter 3** for further information about staining protocol, image capture and image analysis. For this Chapter, slides were incubated with primary antibodies against: eNOS (610297, BD, USA), Phospho-eNOS Ser^{1177} (peNOS Ser^{1177}) (07-428-I, Merck), NAD(P)H-oxidase subunit NOX2 (kind gift from Prof Mark Quinn, Montana State University), tumor suppressor protein p53 (p53) (Ab26, Abcam, UK), cyclin-dependent kinase inhibitor 1 (p21) (Ab109520, Abcam, UK), cyclin-dependent kinase inhibitor 2A (p16) (Abc51243, Abcam, UK), nitrotyrosine (Ab7048, Abcam, UK), ET-1 (PA3-067, Thermo Fisher Scientific, USA), NFκB (NB100-56712, Novus, UK). Importantly for this Chapter, EC protein expression data are reported as ratios to human umbilical vein endothelia cell (HUVEC) protein expression. A single technician analysed each batch of slides. Technicians were blinded to subject identity during the staining and analysis procedures.

Statistical analysis

All analyses were performed using IBM SPSS statistics for Windows, version 25.0. (Armonk, NY: IBM Corp). A mix-linear model was used to test the changes of vascular

parameters (diameter, mean shear rate etc.) in response to different intensities of handgrip exercise. Differences in vascular function and EC protein expression between patients with obstructive and non-obstructive CAD were determined by *t*-test. Pearson correlation analysis was used to determine relations of interest. Data are presented as mean±SD and statistical significance for all analyses was set at $P \leq 0.05$.

5.4. RESULTS

Relationship between EC protein expression with endothelium-dependent dilation and exercise response

FMD was positively correlated with eNOS Ser^{1177} phosphorylation (r=0.290, P=0.037) (Figure 5.1) and p16 (r=0.426, P=0.002) and p21 protein expression (r=0.307 P=0.027) (Figure 5.2). When eNOS Ser^{1177} phosphorylation was normalized to eNOS content the significant correlation was no longer present (r=0.218, P=0.121) (Table 5.4). No significant association was observed between FMD and eNOS, NOX2, ET-1 and NFκB protein expression or nitrotyrosine abundance in patient ECs (Table 5.2). HE-mediated dilation was not correlated with any of the proteins examined (Table 5.2).

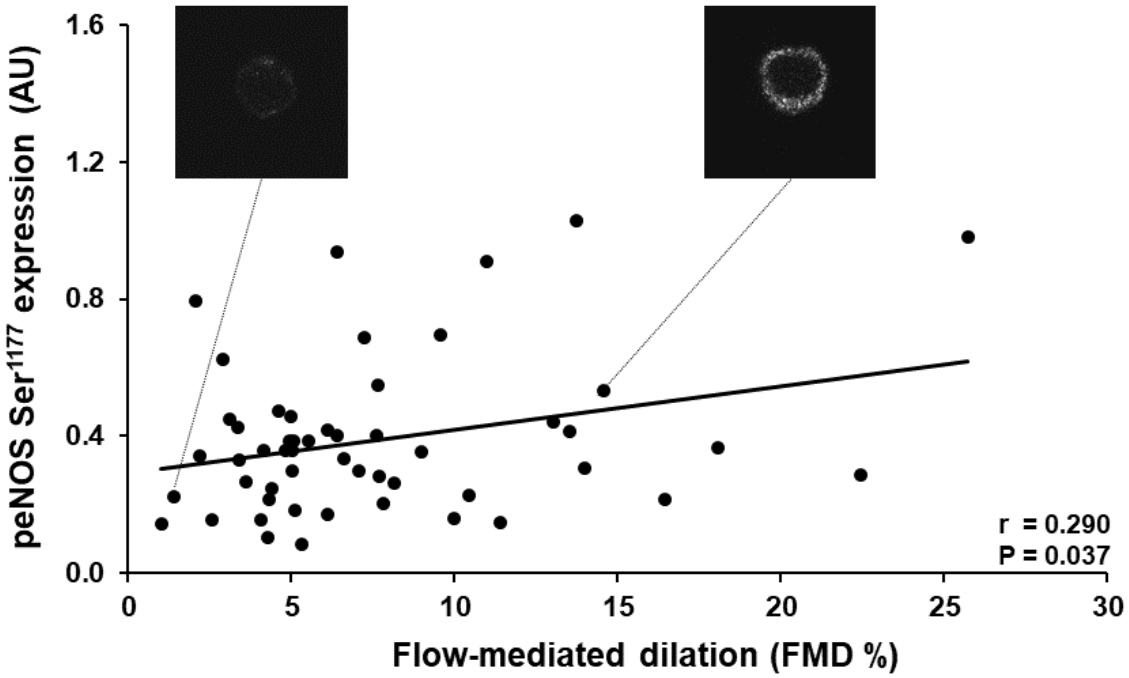
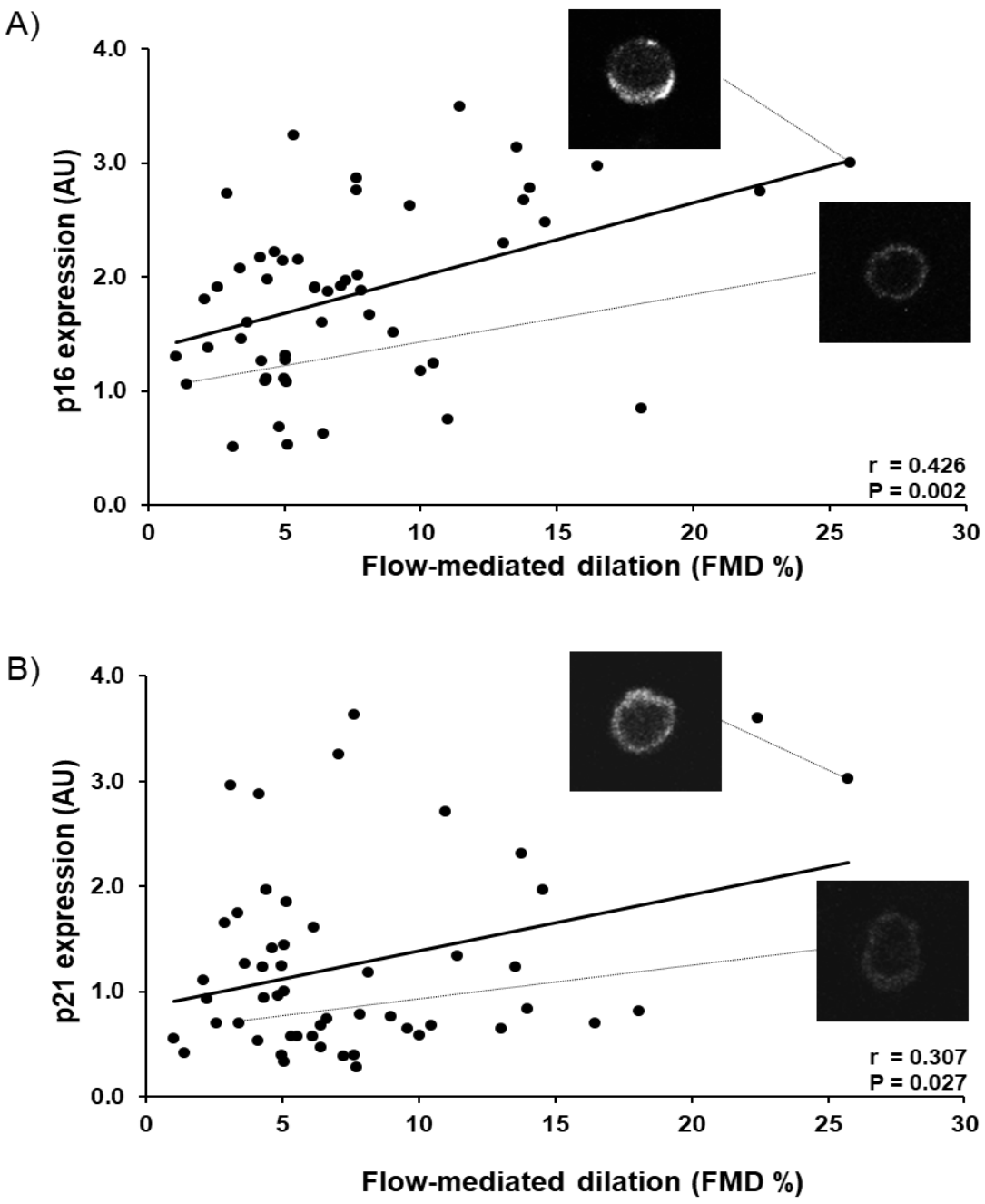


Figure 5.1. Positive correlation between flow-mediated dilation (FMD%) and eNOS Ser^{1177} phosphorylation (peNOS Ser^{1177}) in endothelial cells (EC)s obtained from radial arteries of coronary artery disease patients.

AU: arbitrary units EC protein expression data are reported as ratios to human umbilical vein endothelial cells (HUVEC) protein expression.



<u>Figure 5.2.</u> Positive correlation between flow-mediated dilation (FMD%) with p16 (A) and p21 (B) protein expression in endothelial cells (EC)s obtained from radial arteries of coronary artery disease patients.

AU: arbitrary units EC protein expression data are reported as ratios to human umbilical vein endothelial cells (HUVEC) protein expression; p16: cyclin-dependent kinase inhibitor 2A; p21: cyclin-dependent kinase inhibitor 1.

Table 5.2. Associations between flow-mediated dilation (FMD), vasodilation induced by handgrip exercise (HE%) and endothelial cell protein expression. Pearson correlation coefficient (r) and P value are reported.

	FMD (N=54)	HE% (N=46)
eNOS	r=-0.039, P=0.781	r=0.227, P=0.138
peNOS Ser^{1177} *	r=0.290, P=0.037	r=-0.261, P=0.087
peNOS Ser^{1177}/eNOS	r=0.218, P=0.121	r=-0.252, P=0.099
ET-1	r=0.177, P=0.214	r=-0.159, P=0.308
p16 *	r=0.426, P=0.002	r=-0.29, P=0.852
p21 *	r=0.307, P=0.027	r=-0.207, P=0.178
p53	r=0.108, P=0.447	r=0.049, P=0.752
NT	r=0.209, P=0.140	r=0.231, P=0.136
NOX2	r=0.177, P=0.219	r=-0.190, P=0.222
NFκB	r=0.105, P=0.463	r=-0.17, P=0.915

*Significant association with FMD (*P*<0.05). eNOS: endothelial nitric oxide synthase; peNOS Ser ^{1177}: phospo-eNOS Ser^{1177}; ET-1: endothelin-1, p16: cyclin-dependent kinase inhibitor 2A; p21: cyclin-dependent kinase inhibitor 1; p53: tumor suppressor p53; NT: nitrotyrosine; NOX2: NADPH oxidase subunit NOX2; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

Comparison between patients with obstructive and non-obstructive coronaries

Endothelium-dependent dilation

There was no difference in FMD between the patients with non-obstructive CAD and obstructive CAD (non-obCAD: 8.7±5.9%, obCAD: 7.0±4.7%, P=0.278). Similarly, no difference was observed in baseline diameter (non-obCAD 2.7±0.6 mm, obCAD

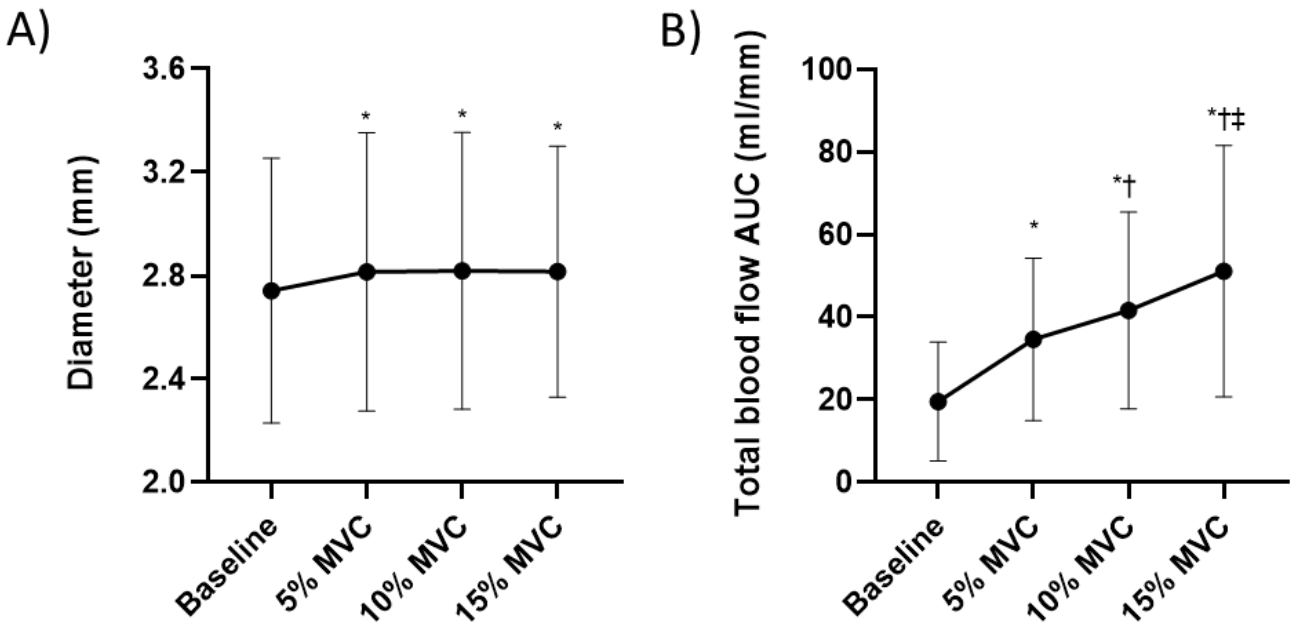
2.8±0.6 mm P=0.372), peak diameter (non-obCAD: 2.9±0.6 mm, obCAD: 3.0±0.6 mm P=0.482), total SRAUC (non-obCAD: 27.5±13.5 $s^{-1}.10^3$, obCAD: 19.9±14.6 $s^{-1}.10^3$, P=0.082) and time to peak (non-obCAD: 60.5±28.1 s, obCAD: 60.3±31.9 s, P=0.985) between the two patient groups.

Radial artery response to handgrip exercise

There was a significant main effect of exercise intensity (P<0.0001) on diameter, blood velocity, total blood flow AUC, anterograde blood flow AUC, retrograde blood flow AUC, mean shear rate, anterograde shear rate AUC and retrograde shear rate AUC. Importantly, there was a stepwise increase (P<0.05) in total blood flow AUC (Figure 5.3B) and mean shear rate (Table 5.3), whereas diameter increased from baseline to 5% MVC, and was then remained elevated throughout exercise (P<0.001) (Figure 5.3A). Detailed data from pairwise comparisons are displayed in Table 5.2. Finally, there was no difference percentage change of diameter during handgrip exercise between patient groups (non-obCAD: 5.4±5.7%, obCAD: 5.7±5.7% P=0.888).

Endothelial cell protein expression

There was no difference in endothelial protein expression or phosphorylation between patients with non-obstructive and obstructive CAD (P>0.05) (Table 5.4).



<u>Figure 5.3.</u> Arterial responses to handgrip exercise.

Results are presented as mean±SD, n=47, *P*<0.05. Arterial diameter (A) and total blood flow (B) when compared before exercise (Baseline) and at 5%, 10% and 15% maximal voluntary contraction (MVC). *Significantly different from baseline, †Significantly different from 5% MVC, ‡Significantly different from 10% MVC.

Table 5.3. Vascular responses of the radial artery at baseline and during handgrip exercise.

	Baseline	5% MVC	10% MVC	15% MVC
Blood Velocity (cm/s)	6.1±5.1	9.5±4.9*	11.5±6.7*†	13.6±7.8*†‡
AUC+ve Flow (ml/min)	23.6±15.7	36.7±21.2*	43.0±24.0*†	52.0±30.9*†‡
AUC-ve Flow (ml/min)	-2.9±3.0	-1.1±1.4*	-0.9±1.2*	-0.6±0.5*†
Mean SR (1/s)	96.9±92.1	142.2±77.5*	173.0±117.1*†	199.2±127.4*†‡
SRAUC+ve (1/s)	108.8±90.0	145.9±76.0*	176.3±115.0*†	201.4±126.4*†‡
SRAUC-ve (1/s)	-12.0±13.2	-3.6±4.2*	-3.3±5.9*	-2.0±2.1*

Results reported as mean±SD, n=47, $P \leq 0.05$. *Significantly different from baseline; †Significantly different from 5% MVC; ‡Significantly different from 10% MVC. AUC+ve Flow: anterograde flow area under the curve; AUC-ve Flow: retrograde flow area under the curve; SR: shear rate; SRAUC+ve anterograde shear rate area under the curve; SRAUC-ve: anterograde shear rate area under the curve; MVC: maximal voluntary contraction.

Table 5.4. Differences in protein expression of endothelial function-related markers in endothelial cells (EC)s between patients with non-obstructive CAD (Non-obCAD) and obstructive CAD (obCAD).

	Non-ob CAD (N=18)	ObCAD (N=36)	P value
eNOS	0.40±0.17	0.51±0.26	P=0.113
peNOS Ser^{1177}	0.39±0.29	0.38±0.19	P=0.895
peNOS Ser^{1177}/eNOS	1.17±0.66	0.98±0.43	P=0.205
ET-1	0.71±0.46	0.76±0.55	P=0.766
p16	1.93±0.75	1.78±0.76	P=0.500
p21	1.06±0.75	1.34±0.94	P=0.284
p53	0.57±0.63	0.56±0.69	P=0.983
NT	0.83±0.33	0.81±0.49	P=0.880
NOX2	0.62±0.31	0.73±0.46	P=0.385
NFκB	0.68±0.35	0.57±0.33	P=0.307

Results are reported as mean±SD (n=54) of arbitrary units (ratio of ECs expression to human umbilical vein endothelial cell (HUVEC) average pixel intensity). eNOS: endothelial nitric oxide synthase; peNOS Ser^{1177}: phospo-eNOS Ser^{1177}; ET-1: ndothelin-1; p16: cyclin-dependent kinase inhibitor 2A; p21: cyclin-dependent kinase inhibitor 1; p53: tumor suppressor p53; NT: nitrotyrosine; NOX2: NADPH oxidase subunit NOX2; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells.

Relationship between endothelium-dependent dilation (FMD) and arterial response to exercise

There was no correlation between FMD and the percentage change in diameter during handgrip exercise (r=-0.015, P=0.985).

Associations among EC protein expression in CAD patients

eNOS Ser^{1177} phosphorylation is related to oxidative stress and senescence markers

There was a significant positive correlation between eNOS Ser^{1177} phosphorylation and NOX2 (r=0.306, P=0.029). When eNOS Ser^{1177} was normalised to eNOS content (peNOS Ser^{1177}/eNOS), the correlation with NOX2 was close to significant (r=0.271, P=0.055). eNOS Ser^{1177} phosphorylation was also correlated with the expression of p21 (r=0.269, P=0.049) (Figure 5.4). eNOS Ser^{1177} phosphorylation was not associated with any other EC protein (Table 5.5). eNOS content was also not associated with any other EC protein (Table 5.5).

Relation between NFκB expression with vasoconstriction and oxidative stress

A strong positive correlation was observed between ET-1 and inflammatory marker NFκB (r=0.633, P<0.0001). A weak correlation was also reported between the expression of NOX2 and NFκB (r=0.283, P=0.046) (Figure 5.4). NFκB was not associated with any other EC protein (Table 5.5).

Senescence markers are related to oxidative stress

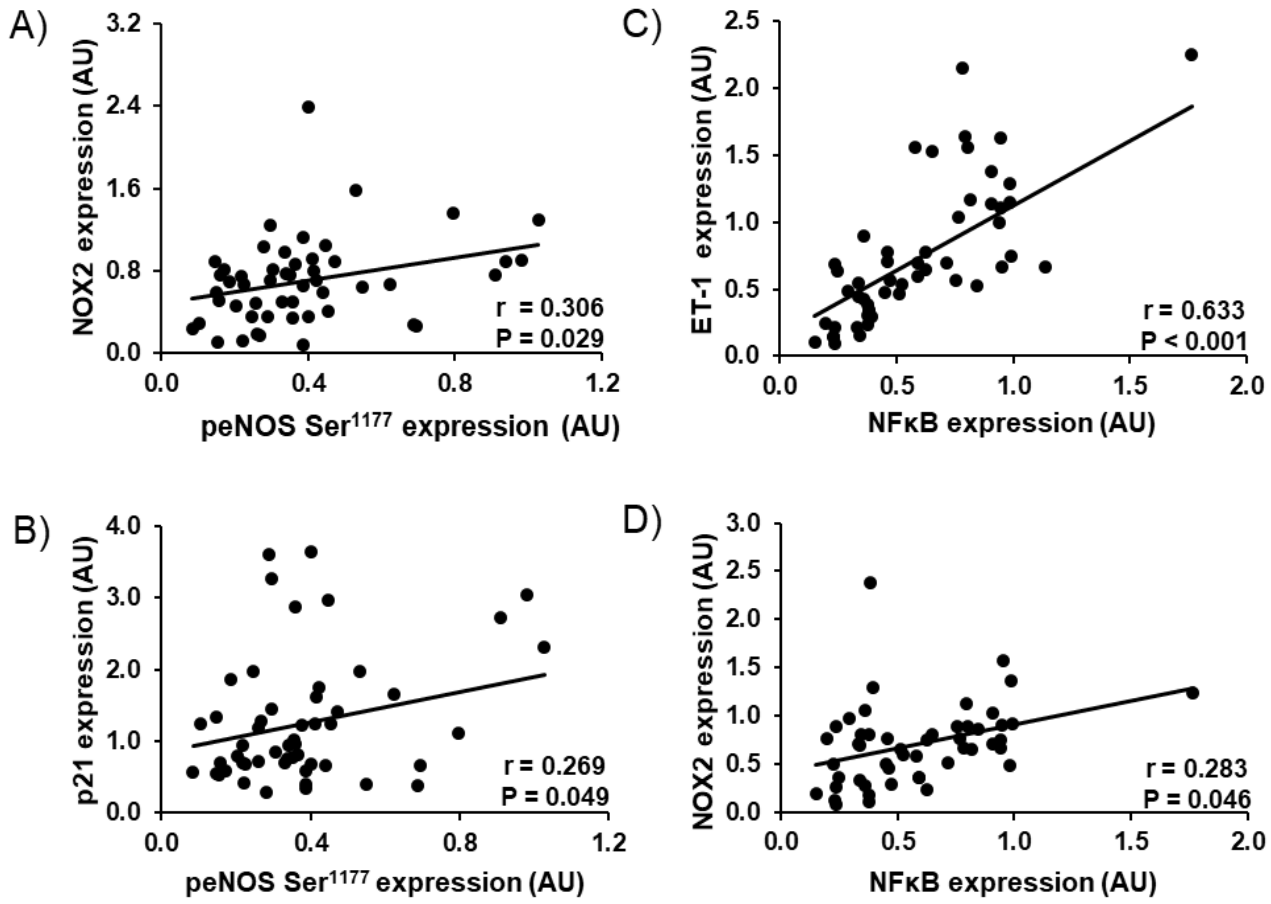
The expression of p16 (r=0.638, P<0.0001) p21 (r=0.308, P=0.025) and p53 (r=0.434, P=0.001) in ECs were positively correlated with the expression of nitrotyrosine. In addition, the expression of NOX2 was positively associated with the expression of p21(r=0.423, P=0.002) and p53 (r=0.459, P=0.001). Finally, there was a strong positive correlation between the expression of the senescence markers p21 and p53 (r=0.598, P<0.0001). These correlations are presented in Figure 5.5. The senescence

and oxidative stress markers were not associated with any other EC proteins (Table 5.5).

Table 5.5. Associations between endothelial cell protein expression in CAD patients (n=54). Pearson correlation coefficient (r) and P value are reported. Significant associations (*P*<0.05) were highlighted in bold.

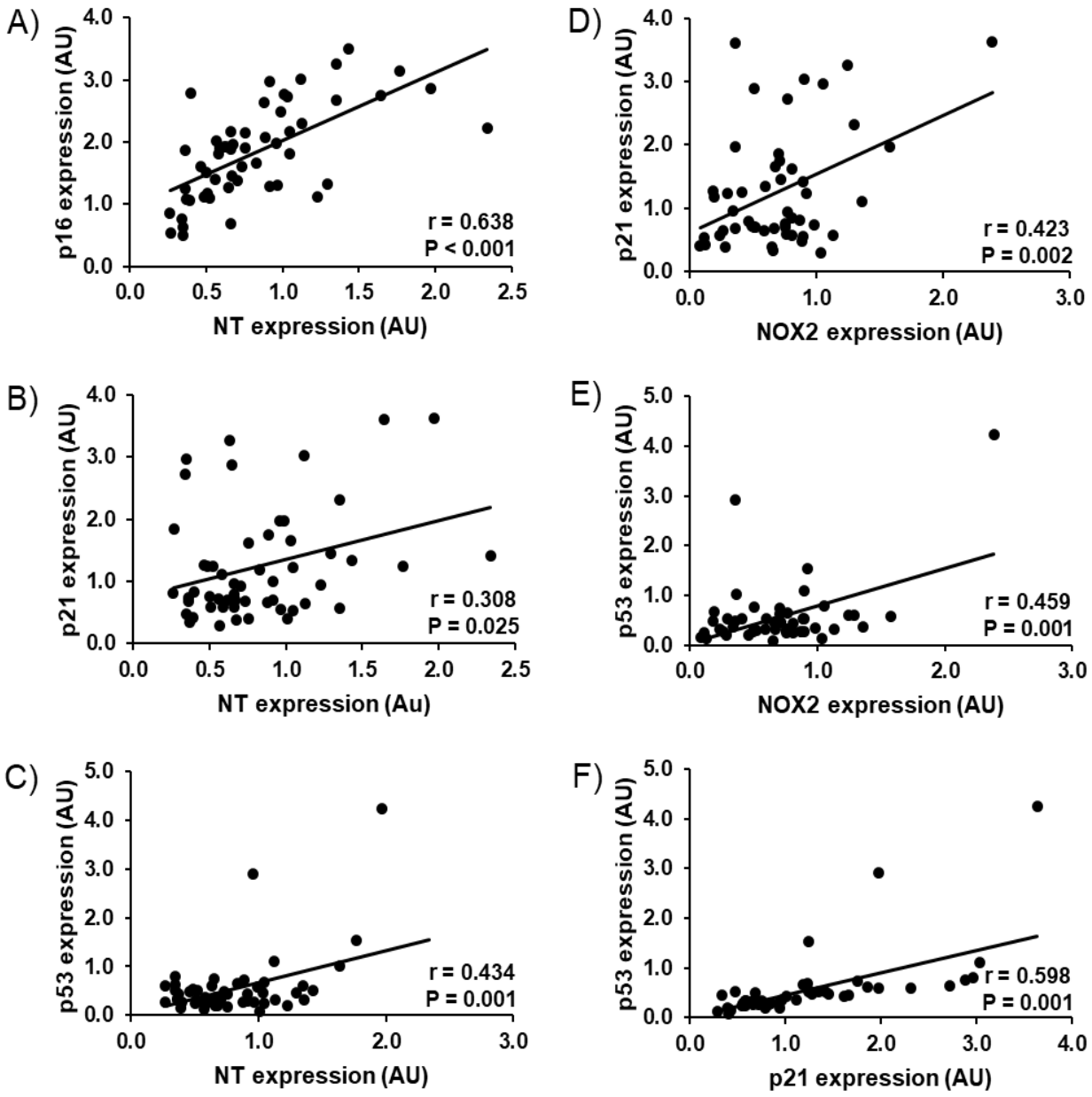
	eNOS	peNOS Ser^{1177}	peNOS Ser^{1177}/eNOS	ET-1	p16	p21	p53	NT	NOX2	NFκB
eNOS		r=0.120	**r=-0.446**	r=-0.069	r=-0.229	r=-0.042	r=0.084	r=-0.124	r=0.005	r=0.074
		P=0.387	**P=0.001**	P=0.623	P=0.099	P=0.763	P=0.544	P=0.377	P=0.972	P=0.597
peNOS Ser^{1177}	r=0.120		**r=0.706**	r=0.222	r=0.075	**r=0.269**	r=0.067	r=0.044	**r=0.306**	r=0.115
	P=0.387		**p<0.001**	P=0.111	P=0.595	**P=0.049**	P=0.630	P=0.752	**P=0.029**	P=0.414
peNOS Ser^{1177}/eNOS	**r=-0.446**	**r=0.706**		r=0.182	r=0.079	r=0.202	r=0.114	r=0.091	r=0.271	r=0.083
	P=0.001	**p<0.001**		P=0.193	P=0.576	P=0.143	P=0.413	P=0.517	P=0.055	P=0.556
ET-1	r=-0.069	r=0.222	r=0.182		r=0.080	r=0.020	r=0.041	r=0.169	r=0.157	**r=0.633**
	P=0.623	P=0.111	P=0.193		P=0.573	P=0.889	P=0.772	P=0.627	P=0.277	**P<0.001**
p16	r=-0.229	r=0.075	r=0.079	r=0.080		r=0.106	r=0.225	**r=0.638**	r=0.132	r=0.042
	P=0.099	P=0.595	P=0.576	P=0.573		P=0.450	P=0.106	**P<0.001**	P=0.362	P=0.768
p21	r=0.042	**r=0.269**	r=0.202	r=0.020	r=0.106		**r=0.598**	**r=0.308**	**r=0.423**	r=-0.065
	P=0.763	**P=0.049**	P=0.143	P=0.889	P=0.450		**P<0.001**	**P=0.025**	**P=0.002**	P=0.646
p53	r=0.084	r=0.067	r=0.114	r=0.041	r=0.225	**r=0.598**		**r=0.434**	**r=0.459**	r=0.035
	P=0.544	P=0.630	P=0.413	P=0.772	P=0.106	**P<0.001**		**P=0.001**	**P=0.001**	P=0.806
NT	r=-0.124	r=0.044	r=0.091	r=0.169	**r=0.638**	**r=0.308**	**r=0.434**		r=0.228	r=0.127
	P=0.377	P=0.752	P=0.517	P=0.627	**P<0.001**	**P=0.025**	**P=0.001**		P=0.102	P=0.370
NOX2	r=0.005	**r=0.306**	r=0.271	r=0.157	r=0.132	**r=0.423**	**r=0.459**	r=0.228		**r=0.283**
	P=0.972	**P=0.029**	P=0.055	P=0.277	P=0.362	**P=0.002**	**P=0.001**	P=0.102		**P=0.046**
NFκB	r=0.074	r=0.115	r=0.083	**r=0.633**	r=0.042	r=-0.065	r=0.035	r=0.127	**r=0.283**	
	P=0.597	P=0.414	P=0.556	**P<0.001**	P=0.768	P=0.646	P=0.806	P=0.370	**P=0.046**	

eNOS: endothelial nitric oxide synthase; peNOS Ser^{1177}: phospo-eNOS Ser^{1177}; ET-1: endothelin-1; p16: cyclin-dependent kinase inhibitor 2A; p21: cyclin-dependent kinase inhibitor 1; p53: tumor suppressor p53; NT: nitrotyrosine; NOX2: NADPH oxidase subunit NOX2; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells



<u>Figure 5.4.</u> Positive relations between eNOS Ser^{1177} phosphorylation with NOX2 (A) and senescence marker p21 (B) of endothelial cells (EC)s obtained from radial artery of coronary artery disease patients. NFκB is correlated with ET-1 (C) and NOX2 (D).

AU: arbitrary units EC protein expression data are reported as ratios to human umbilical vein endothelial cells (HUVEC) protein expression; NOX2: NADPHoxidase subunit 2; p21: cyclin-dependent kinase inhibitor 1; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; ET-1: endothelin-1; peNOS: phospho-endothelial nitric oxide synthase.



<u>Figure 5.5.</u> Positive correlations between senescence and oxidative stress expression of endothelial cells (EC)s obtained from radial artery of coronary artery disease patients. NT expression is related to p16 (A), p21 (B) and p53 (C), NOX2 expression is correlated to p21 (D) and p53 (E), and p21 expression is associated with p53 (F).

AU: arbitrary units EC protein expression data are reported as ratios to human umbilical vein endothelial cells (HUVEC) protein expression; NT: nitrotyrosine; p16: Cyclin-dependent

kinase inhibitor 2A; p21: cyclin-dependent kinase inhibitor 1; p53: tumor protein 53; NOX2: NADPHoxidase subunit 2.

5.5. DISCUSSION

To our knowledge the current study is the first to assess relationships between endothelium-dependent vasodilation in the radial artery and the expression of atherogenic risk modulating proteins in vascular ECs isolated from the radial artery of patients with CAD. The key novel findings were that endothelium-dependent dilation, assessed using FMD, was positively associated with eNOS Ser^{1177} phosphorylation seen in the isolated ECs. In addition, positive associations were observed between FMD and the protein expression of the senescence markers p16 and p21. Interestingly, markers of oxidative stress (NOX2 and nitrotyrosine), inflammation (NFκB) and vasoconstriction (ET-1) in the isolated ECs were not associated with endothelium-dependent vasodilation. Arterial response to exercise was not correlated with eNOS content and/or activation of any other atherogenic risk modulating protein. This finding, together with the absence of correlation between FMD and exercise vasodilation, may suggest that different mechanisms underpin their responses. Whether one is more clinically relevant, or if they provide additive information regarding cardiovascular health and future risk remains to be determined. In addition, none of the atherogenic risk–modulating proteins assessed were different between patients with non-obstructive and obstructive CAD or were associated with vasodilation measured in the radial artery in response to handgrip exercise. Finally, we present a number of associations between the expression of EC proteins which add further weight to the hypothesis that reduced NO bioavailability, elevated oxidative stress, vasoconstriction, inflammation and EC senescence are part of a

vicious cycle contributing to endothelial dysfunction and the development of a pro-atherogenic environment.

Association between endothelium-dependent dilation (FMD) and proteins involved in NO production and scavenging

Increased production of reactive oxygen species within the vasculature is a characteristic feature of CVD, including CAD [367]. In particular, production of superoxide anions promotes the development of atherosclerosis by quenching NO and activating pathways that modulate vessel remodelling and plaque stability [368]. In humans with early coronary atherosclerosis, but not diagnosed CAD (<30% stenosis), coronary endothelial dysfunction has been shown to be associated with elevated release of isoprostanes, a marker of oxidative stress [369]. Furthermore, mRNA expression of NOX2, the catalytic subunit of the NAD(P)Hoxidase complex, has been shown to be elevated in coronary arteries from CAD patients compared to controls undergoing heart transplantation [370]. Together these studies suggest elevated oxidative stress may contribute, mediated through NAD(P)Hoxidase to the development of endothelial dysfunction and CAD. However, data from the present study, in a group of patients with established CAD (>30% stenosis), demonstrated that neither endothelial NOX2 protein content or nitrotyrosine content, a marker of oxidative stress, were associated with endothelium-dependent vasodilation. These findings also contrast data from Donato *et al.* (2007) where age-associated reductions in endothelium-dependent vasodilation, measured through FMD, were associated with increased brachial artery endothelial nitrotyrosine content and the protein content of subunit p47phox of NAD(P)Hoxidase [88].

Endothelial NO produced by eNOS exerts multiple antihypertensive, antithrombotic and antiatherogenic effects [190]. Consistent with the anti-atherosclerotic role of eNOS, genetic

disruption of eNOS in ApoE-knockout mice enhances atherosclerosis [7]. However, human studies have shown that endothelial eNOS protein content is not altered in obesity [77] or ageing [76], and age-associated reductions in endothelium-dependent dilation are not associated with eNOS protein content, notwithstanding the impaired NO bioavailability that occurs in each of these phenotypes. In addition, coronary endothelial dysfunction in humans is not associated with reduced basal NO production, in early coronary atherosclerosis [369]. Together these data suggest that reduced NO production does not contribute to the endothelial dysfunction which is apparent in individuals with risk factors and CAD. These findings are supported by the current study, where eNOS content was not associated with endothelium-dependent vasodilation (FMD). peNOS Ser^{1177}/eNOS ratio, reflecting the ability of ECs to phosphorylate the available eNOS, was also not associated with endothelium dependent dilation. However, total eNOS Ser^{1177} phosphorylation, reflecting the overall activation of eNOS had a positive association with endothelium-dependent vasodilation (FMD). This finding is in contrast with observations in healthy ageing, where age-associated reductions in endothelium-dependent vasodilation were not associated with eNOS Ser^{1177} phosphorylation [76]. The observation that total eNOS Ser^{1177} phosphorylation was associated with endothelium-dependent dilation but eNOS content and peNOS Ser^{1177}/eNOS ratio were not, may suggest that together reduced eNOS content and ability to phosphorylate the available eNOS combine to result in reduced overall eNOS phosphorylation and therefore activation. The overall phosphorylation of eNOS is relevant, as it is the overall activation of the enzyme not the content or ability to phosphorylate eNOS which will determine NO production [371].

Together, the observations that NT content and NOX2 expression are not associated with endothelium-dependent dilation while eNOS Ser^{1177} phosphorylation may suggest a

difference in the regulation of endothelium-dependent dilation between patients with established CAD and those without a clinical diagnosis. Data from individuals without CAD suggests that elevated oxidative stress, but not impaired NO production, plays a role in the development of vascular dysfunction [8, 76, 369, 372]. Our data also seem to suggest that further elevations in oxidative stress do not relate to progressive declines in endothelium-dependent vasodilation in patients with diagnosed CAD, with activation of eNOS by Ser^{1177} phosphorylation becoming increasingly important to maintain NO production. In support of this, Hambrecht *et al.* [41] showed in CAD patients, that the post training basal eNOS Ser^{1177} phosphorylation (left internal mammary artery) was associated with the change in endothelium-dependent dilation, as assessed through acetylcholine infusion. Furthermore, a number of drugs used to treat CVD and improve endothelium-dependent dilation, oestrogens [373], statins [374], and peroxisome proliferator activated receptors agonists [341], also are able to activate eNOS by stimulating eNOS Ser^{1177} phosphorylation [375].

A positive association was found between endothelial eNOS Ser^{1177} phosphorylation and NOX2 protein content. It has previously been shown that reactive oxygen species can modulate NO production by influencing eNOS activity [368], specifically H_2O_2 has been shown to increase phosphorylation of eNOS at Ser^{1177} [82]. As such, the association between eNOS Ser^{1177} phosphorylation and NOX2 could reflect an attempt to increase NO production and overcome elevated NO scavenging by superoxide, as has been hypothesised in ageing [88, 127].

Association of endothelium-dependent dilation (FMD) with markers of endothelial senescence

Cellular senescence, a stress-response resulting in irreversible growth arrest of a cell, is emerging as a potential driver of endothelial dysfunction and the development of atherosclerosis [128]. The accumulation of senescent cells, and the pro-inflammatory and pro-oxidative senescence associated secretory phenotype (SASP) of these cells have been proposed as potential mediators of the adverse effects of senescence [133, 134]. Importantly, the tumor suppressor pathways p53/p21 and p16 have been shown to be activated by cellular stressors such as DNA damage, dysfunctional telomeres, oxidative stress, or metabolic stimuli, activating cell cycle arrest [128, 376]. In support of the role of senescence in the development of atherosclerosis, p16 expression has been shown to be increased in atherosclerotic lesions in mice [138]. However, direct evidence associating senescence with endothelial function in humans is limited. A recent study showed that age-associated reductions in endothelium-dependent dilation were negatively associated with endothelial p53, p21 and p16 protein content [126]. However, data from the current study shows that endothelium-dependent vasodilation was positively associated with endothelial protein content of p21 and p16 in CAD patients.

Both associations were unexpected and, given the lack of human studies investigating vascular senescence in CAD, we cannot fully explain these observations. However, although the prevailing hypothesis is that endothelial senescence leads to vascular dysfunction, studies in murine models of high-fat diet induced atherogenesis have shown that knockout of p53 [377] and p21 [378], resulted in greater severity of atherosclerotic lesions. As such, the role of senescence within the vasculature may depend on the stage of the progression of atherosclerosis [133, 135], with senescence initially playing a protective

role. It has been proposed that senescent cells may delay the atherosclerotic process in the beginning as they prevent cell growth by activating cell arrest and/or perhaps apoptosis pathways [377]. Future studies should test the causal contribution of senescence at different stages of CAD pathology.

All three markers of senescence (p53, p21 and p16) were associated with EC nitrotyrosine abundance, and p52 and p21 protein content were associated with NOX2 protein content. These associations are supported by previous work showing oxidative stress leads to stress induced senescence, through the activation of p53/p21 and p16 pathways [133, 138, 379]. Surprisingly, endothelial NFκB was not associated with any marker of senescence. Importantly, NFκB is known to initiate and maintain the SASP, upregulating inflammatory mediators and further production of reactive oxidative species [133, 137].

EC p21 protein content was associated with eNOS Ser^{1177} phosphorylation. This finding is also in contrast to previous data surrounding the role of senescence in ECs. *In vitro* data from human aortic ECs where senescence was induced by inhibiting telomere function shows that eNOS protein content and activity were reduced [380]. However, the association may provide a link between elevated expression of senescence markers and improved endothelium dependent dilation, given eNOS Ser^{1177} phosphorylation was also positively associated with endothelium dependent dilation. The apparent disconnect between *in vitro* and *in vivo* data supports the suggestion that the role of endothelial senescence in CAD should be investigated in future studies, evaluating its contribution to the development of CAD at different stages.

Pro-inflammatory transcription factor NFκB is associated with NOX2 and vasoconstrictor ET-1

NFκB is a key transcription factor in the regulation of pro-inflammatory markers [381]. Endothelial expression NFκB had a positive correlation with NOX2 protein content. This observation is supported by evidence which suggests NFκB may be an important link between inflammation and oxidative stress [76, 382]. Indeed, previous work demonstrates the potential for NAD(P)Hox dependent induction of NFκB [116]. Similarly, NFκB inhibition resulted in reduced NOX2 expression and improved endothelium-dependent dilation in obese subjects [117], suggesting a vicious cycle between inflammation and oxidative stress.

The expression of the potent vasoconstrictor ET-1 had a strong positive correlation with NFκB expression. ET-1 has been proposed to affect endothelial function via inflammatory pathways [119, 120], as cardiac overexpression of ET-1 in mice is associated with increased activation of NFκB [383]. In turn, NFκB stimulates ET-1 expression [119, 384]. As such, our data further highlight the critical role of NFκB as a key regulator in the development of endothelial dysfunction in CAD.

Endothelium-dependent dilation, exercise-induced vasodilation and EC protein expression is unchanged through CAD progression

An additional aim of the study was to examine whether FMD, exercise-induced dilation and the EC expression of atherogenic risk-modulating proteins was different between CAD patients with obstructed and non-obstructed coronaries, to see if such changes could explain differences in disease progression. We demonstrated that neither FMD or diameter change to exercise, nor expression of endothelial proteins were different between CAD patients with obstructed and non-obstructed coronaries. To our knowledge this is the first study to look at

these factors in CAD patients with different levels of coronary artery stenosis (obCAD >70% stenosis vs. non-obCAD 30-70% stenosis). Previous work has focused on comparing CAD patients with documented stenosis with either healthy individuals or patients with angiographically normal coronaries. Such work has demonstrated that CAD patients (≥30% stenosis) have lower FMD than patients with angiographically smooth coronaries and healthy controls (age:17-36 years, ≤1 CV risk factor) [385], and that EC protein expression switches towards a pro-inflammatory and pro-oxidative phenotype in patients with CAD [84, 370]. Similarly, exercise-induced constriction in coronaries has been observed in atherosclerotic lesions of CAD patients, when compared to healthy controls [328, 329] or angiographically smooth arteries [326]. This may suggest that alternative mechanisms, for example duration of endothelial dysfunction, are more important in determining progression of CAD in those with a clinical diagnosis.

FMD does not predict arterial response to exercise

Interestingly, FMD was not correlated with the vasodilation induced during handgrip exercise, and unlike FMD, the arterial response to exercise was not associated with any of the atherogenic risk modulating proteins assessed. This may be due to the different mechanisms responsible for dilation in response to the two tests. The FMD response is determined by a large and transient increase in shear stress, whereas a more gradual, sustained increase in shear stress is observed during exercise [386]. In addition, FMD response is known to be endothelium-dependent and primarily mediated to a large extent though endothelial NO production [346]. In contrast, regulation of blood flow during exercise is more complex, involving a number of mechanisms such as transmural pressure and vasoactive compounds (NO, prostacyclin, endothelial-derived relaxing factor (EDRF)) with multiple interactions and redundancy [25, 50, 358, 359]. Our data indicated that eNOS

Ser^{1177} phosphorylation was positively correlated with FMD, but not with arterial response to exercise, which may support the different mechanisms involved among these two tests. However, it is worth noting that previous evidence indicating upregulation of both eNOS content and Ser^{1177} activation in arteries of CAD patients following 8 weeks of exercise training, supporting the association between NO-production catalysing enzyme and exercise response [147]. Given this contrast, further studies should be conducted including healthy subjects with intact endothelial layer to clarify whether arterial response to exercise is associating with higher eNOS content and/or activation. Taking into consideration our findings, we believe that the absence of an association between FMD and exercise response in CAD patients, may reflect the different mechanisms that contribute to these physiological functions.

This study has a number of limitations. Firstly, vascular measurements were taken on the day of catheterization and although patients were instructed to follow the guidelines for FMD testing in regards to food, exercise, alcohol and smoking, they continued their medications as instructed by their consultant, as discontinuation of medications could have increased the risk for vascular complications during and/or after the procedure (i.e. thrombosis). Such medications could have affected the *in vivo* measurements of vascular function (perhaps relatively higher FMD and vasodilation to exercise), but it is unlikely to affect the protein expression of ECs. This may affect the correlations among the *in vivo* measurements and EC protein expression. Importantly, previous studies evaluating radial artery FMD in CAD patients have reported similar responses prior to catheterization [49, 163], supporting that our FMD data are within the normal range. In addition, although we performed Pearson correlations between multiple variables, we did not use adjusted the P value using Bonferroni corrections. This may have affected the outcomes, however, given the small data

set (54 patients) and the nature of our study which aimed to explore associations, we believed that Bonferroni corrections would have been too conservative and perhaps results in losing some associations with clinical significance.

Summary and Conclusions

The results of this study provide new insight into the molecular events underlying the development of endothelial dysfunction in patients with CAD. Importantly, we demonstrated that endothelium-dependent dilation in patients with diagnosed CAD was positively associated with eNOS Ser^{1177} phosphorylation in the harvested ECs. Conversely, endothelium-dependent dilation was not associated with markers of oxidative stress and inflammation in the ECs (nitrotyrosine content and protein content of NOX2, NFκB and ET-1). The data suggests that there is a difference in the regulation of endothelium-dependent vasodilation measured *in vivo* in the radial artery between patients with CAD and those without a clinical diagnosis. The data in the isolated ECs suggest that patients with CAD manage to maintain vasodilation by activation of eNOS by means of Ser^{1177} phosphorylation. This study also revealed a positive association between *in vivo* endothelium-dependent vasodilation and the protein expression of senescence markers p16 and p21 in the isolated ECs. This observation was unexpected and suggests that future work should investigate the role of these senescence markers in the progression of CAD.

CHAPTER 6 - Impact of catherization on shear-mediated arterial dilation in healthy men.

6.1. ABSTRACT

Animal studies have shown that endothelial denudation abolishes vasodilation in response to increased shear stress. Interestingly, reduced but not abolished, shear-mediated dilation has been reported in coronary artery disease (CAD) patients following endothelial denudation. However, it is not known whether this resulted from *a priori* endothelial dysfunction in this diseased population. In this study we aimed to evaluate shear-mediated dilation following catheterization in healthy young men. Twenty-six (age: 24.4±3.8 years, BMI: 24.3±2.8 $kg.m^{-2}$, VO_{2peak}: 50.5±8.8 ml/kg/min) healthy males underwent unilateral transradial catheterization. Shear-mediated dilation of both radial arteries was measured using flow-mediated dilation (FMD) pre, and 7 days post, catheterization. FMD was reduced in the catheterized arm [9.3±4.1% to 4.3±4.1% (P<0.001)] 7 days post-catheterization, whereas no change was observed in the control arm [8.4±3.8% to 7.3±3.8% (P=0.168)]. FMD was completely abolished in the catheterized arm in 5 participants. Baseline diameter (P=0.001) and peak diameter during FMD (P=0.035) were increased in the catheterized arm 7 days post-catheterization (baseline: 2.3±0.3 to 2.6±0.2mm, P<0.001, peak: 2.5±0.3 to 2.7±0.3mm, P=0.001), with no change in the control arm (baseline: 2.3±0.3 to 2.3±0.3mm, P=0.288, peak: 2.5±0.3 to 2.5±0.3mm, P=0.608). This is the first study to provide direct evidence of impaired shear-mediated dilation following catheterization in young trained individuals with fully intact *a priori* endothelial function. Abolition of FMD in $1/5^{th}$ of participants suggests that the endothelium plays an essential role in shear-mediated dilation in healthy individuals, and perhaps the absence of FMD abolition in CAD patients is related to a *priori* endothelial dysfunction.

6.2. INTRODUCTION

The vascular endothelium plays a fundamental role in the regulation of vascular tone [25, 54]. Previous animal work has demonstrated that, following endothelial denudation, arteries no longer respond to increased blood flow [16] or exercise [15], reflecting the critical role of ECs in the regulation of vascular tone. However, Dawson *et al.* [46] showed depressed, but not abolished, radial artery shear mediated dilation following catheterization. When combined with studies showing that flow mediated dilation (FMD) was not completely abolished by endothelial nitric oxide synthase inhibition [346, 387-389], this raises the question of the extent to which FMD responses are dependent upon an intact and functional endothelium in humans. However, the work of Dawson *et al.* [46] was conducted in CAD patients where *a priori* endothelial dysfunction was likely apparent [385].

In the current study, we aimed to assess radial artery shear-mediated dilation following catheterization in healthy, young trained males. Young trained individuals were chosen as a model in whom the endothelial layer is healthy and functional. FMD was used to assess shear-mediated arterial responses in both radial arteries before, and 7 days post catheterization. We hypothesised that catheterization would either preserve or impair shear-mediated dilation in these healthy men. If shear-induced dilation was preserved in arteries with a fully functional endothelial layer, this may be a justification to apply pre-rehabilitation (exercise training) in patients prior to coronary interventions to reduce the endothelial dysfunction related to arterial injury. In contrast, if vasodilation was impaired in apparently healthy arteries, similar to CAD, this may suggest that *priori* arterial heath does not affect catheterization-induced damage.

6.3. METHODS

Participants

Thirty-one healthy young (<35 years), trained males (≥150 minutes of moderate-intensity or ≥75 minutes of high-intensity exercise per week) with a BMI <32 were recruited. This threshold (BMI<32) was selected in order to avoid obese participants (CVD risk factor) but do not exclude healthy active males with increased muscle development due to misclassification [390]. Participants were non-smokers and were free of cardiovascular disease (CVD), or CVD risk factors such as diabetes, hypertension or hypercholesterolemia. None reported taking medications or any drugs that would impact vascular function. Informed consent was gained from all participants prior to the experimental procedures. The study conformed to the Declaration of Helsinki, and ethical approval was obtained from the Liverpool East NHS Research Ethics Committee (18/NW/0428).

Study design

Participants attended the cardiovascular lab at Liverpool John Moores University on three occasions: a) baseline, b) catheterization and c) follow-up. All experimental procedures were conducted between 7am and 1pm, in a quiet temperature-controlled room, and participants were fasted overnight and instructed to abstain from caffeine (>8h), alcohol and vigorous exercise (>24 hours) before each visit, in accordance with current guidelines [343]. During the baseline visit, radial artery (RA) shear-mediated dilation was assessed in both arms using FMD. Following FMD, peak oxygen consumption (VO_{2peak}) during maximal graded exercise on a cycle ergometer (Lode Excalibur Sport Cycle Ergometer, The Netherlands) was also assessed, using a gas analysis system (MOXUS Metabolic Cart (AEI Technology, USA), as descripted in **Chapter 3**. At least 48h following baseline, participants attended the catheterization trial, where a transradial catheter was inserted into the participants RA

(CATH arm). Seven days following catheterization participants attended the lab for the follow-up visit, where FMD was again assessed in the catheterized arm, while the contralateral arm was used as an internal control (CON arm).

Transradial catheterization

An 18-20-gauge catheter (0.9-1.2 mm diameter, 8-10 cm length) (leadercath, Vygon, UK) was inserted into the right RA (15.4% via left RA), as descripted in **Chapter 3**.

Bilateral radial FMD

Following at least 10 minutes supine rest, blood pressure and heart rate were measured using an automated sphygmomanometer (GE Pro 300V2, Dinamap, Tampa, FL, USA). RA shear-mediated dilation was then measured in both arms using FMD, as descripted in **Chapter 3**. The same ultrasounds and sonographers were used between visits, and within participants.

Statistical analysis

All analyses were performed using IBM SPSS statistics for Windows, version 25.0. Armonk, NY: IBM Corp. Allometric scaling was performed to control for differences in baseline diameter [352], and a mix-linear model with covariate control for scaled baseline diameter, was used to determine the main effect of time and arm. A mix-linear model was also used to analyze the differences in baseline diameter, peak diameter, time to peak and SRAUC, during FMD. Pairwise comparisons were performed when significant main or interaction effects were detected, using Fisher's least significant difference (LSD) test. Pearson

correlation analysis was used to determine relations of interest. Paired t-tests were used to assess differences in hemodynamic parameters, pre- and post-catheterization. Results are presented as mean±SD, and significance was set at $P \leq 0.05$.

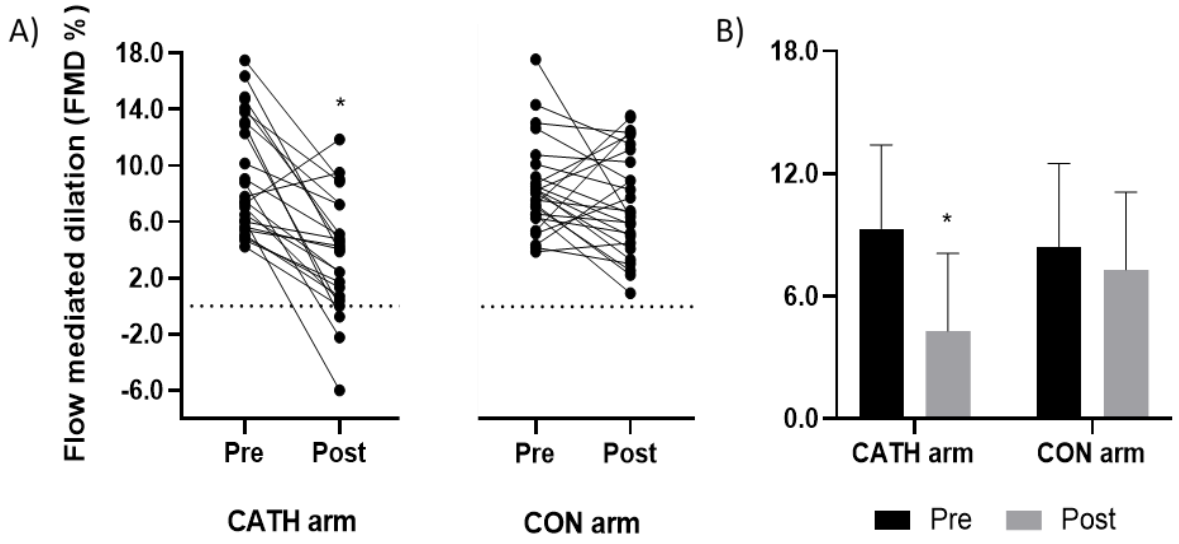
6.4. RESULTS

Twenty-six participants (age: 24.4±3.8 years, body mass index (BMI): 24.3±2.8 $kg.m^{-2}$) completed all three experimental visits. All participants trained for at least ≥150 minutes of moderate-intensity or ≥75 minutes of high-intensity exercise per week. Mean peak oxygen consumption (VO_{2peak}) was 50.5±8.8 ml/kg/min. Out of 104 FMD scans (2 arms, pre- and post-catheterization, 26 participants), 3 scans were excluded from further analysis (2 temporary radial artery spasm in the catheterized artery, 1 poor-quality scan).

Effects of catheterization on radial FMD

There was a significant interaction (time*arm) for FMD, when controlling for baseline diameter (P=0.004) (Figure 6.1), and without baseline diameter normalization (P<0.001). There was no significant difference in FMD between arms, pre-catheterization (P=0.303). There was a significant reduction in FMD in the catheterized arm pre- to 7 days post-catheterization (9.3±4.1% to 4.3±4.1%; P<0.001). FMD was completely abolished in 5 and was <1% in a further 2 of the 26 participants. In contrast, FMD in the catheterized artery was not abolished (<1%) in any of CAD patients **(Chapter 4).** There was no significant change in FMD in the control arm (8.4±3.8% to 7.3±3.8%; P=0.168), SRAUC (time*arm P=0.189) and time to peak (time*arm P=0.664) were not different between arms or pre- to post-catheterization (Table 6.1).

To isolate the magnitude of local FMD change as a result of catheterization we calculated the FMD change in the catheterized arm (pre- to post-catheterization), then subtracted the change in FMD in the control arm (pre- to post-catheterization) (Figure 6.2). There was a significant reduction in FMD (>5%) as a result of catheterization in 9 out of 23 participants. This FMD change was not correlated with the baseline diameter in the catheterized artery prior to catheterization (r=0.300, P=0.164), or with participants' age (r=0.217, P=0.320), or BMI (r=-0.244, P=0.263), or VO_{2peak} (r=0.344, P=0.108). Catheters with external diameter of 0.9 mm and 1.2 mm were used in 16 and 7 participants respectively. There was no impact of catheter size on FMD change following catheterization (r=-0.179, P=0.413). Artery-to-sheath ratio (arterial diameter/external diameter of the catheter) was not associated with FMD change following catheterization (r=0.291, P=0.177).



<u>Figure 6.1.</u> Flow-mediated dilation (FMD%) in the catheterized (CATH) and contralateral radial arteries (CON), pre- and 7 days post endothelial disruption (Post).

A) Individual responses and B) Summary data, presented as mean±SD n=26. *Significantly different from Pre (*P*< 0.05).

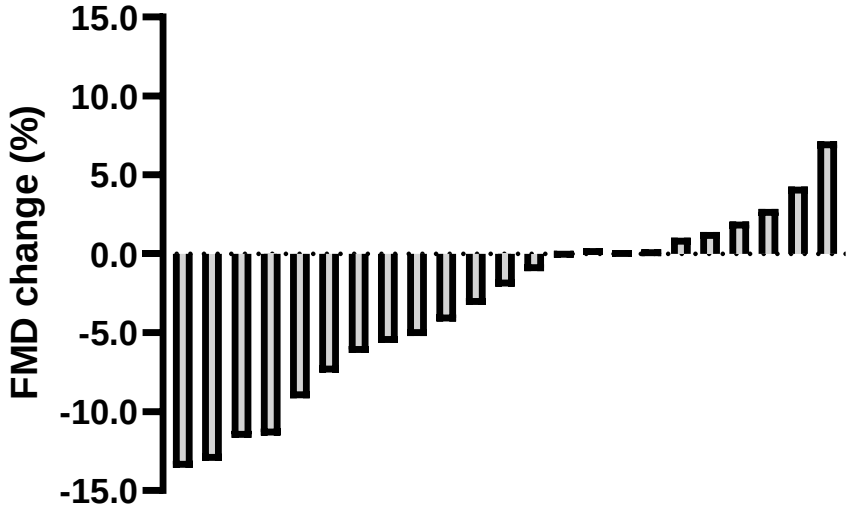


Figure 6.2. Flow-mediated dilation (FMD %) change in the catheterized arm, pre- to post-catheterization, after accounting for change in the control arm, pre- vs post-catheterization (n=23).

Effects of catheterization on arterial diameter

There was a significant interaction effect (time*arm) for baseline diameter (P=0.001) (Table 6.1), demonstrating an increase in baseline diameter in the catheterized arm 7 days post-catheterization, when compared with pre-catheterization (P<0.001) or when compared to the contralateral control arm 7 days post-catheterization (P<0.001). There was no change in the control arm pre- to 7 days post-catheterization (P=0.288). In addition, a significant interaction effect (time*arm) was shown for peak diameter, as assessed during FMD (P=0.035) (Table 6.1). When pairwise comparisons were performed, peak diameter in the catheterized arm was higher 7 days following catheterization compared to pre-catheterization (P=0.001) or in the contralateral control arm (P<0.001), whereas no change in peak diameter was reported in the control arm pre- to post-catheterization (P=0.608).

Table 6.1. Baseline diameter, peak diameter, time to peak and shear rate area under the curve (SRAUC) before (Pre) and at 7 days post-endothelial disruption (Post), in the catheterized (CATH) arm and the contralateral (CON) arm.

	CATH arm		CON arm	
	Pre	**Post**	**Pre**	**Post**
Baseline diameter (mm)	2.32±0.28	2.62±0.28*	2.26±0.23	2.32±0.25†
Peak diameter (mm)	2.54±0.32	2.72±0.31*	2.46±0.26	2.49±0.28†
Time to peak (s)	58.1±27.9	65.5±38.5	54.6±25.27	57.5±27.3
SRAUC (s^{-1} 10^3)	32.9±17.5	27.4±17.8	27.2±14.6	28.7±17.4

Results are presented as mean±SD, n=26, $P<0.05$. *Significantly different from Pre, †Significantly different from CATH arm post-endothelial disruption.

Haemodynamic measurements

There was no change in systolic blood pressure, diastolic blood pressure, mean arterial pressure or heart rate from baseline to follow-up (P>0.05). Data are reported in Table 6.2.

Table 6.2. Resting haemodynamic measurements pre-catheterization (Pre) and 7 days post-endothelial disruption (Post).

	Pre	Post	P value
SBP (mmHg)	116±9	113±7	0.220
DBP (mmHg)	63±7	60±6	0.161
MAP (mmHg)	79±13	77±12	0.089
HR (beats per min)	61±6	62±5	0.435

Results are presented as mean±SD, n=26. SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean blood pressure; HR: heart rate.

6.5. DISCUSSION

The aim of this study was to determine the impact of radial artery catheterization on shear-mediated dilation in healthy, young trained males. We demonstrated that catheterization increased baseline diameter and impaired shear-mediated dilation in the catheterized RA. Indeed, shear-mediated dilation was abolished in almost 1/5th of participants following catheterization. Neither baseline diameter, age, BMI, fitness level (VO_{2peak}), nor catheter size or artery-to-sheath ratio predicted FMD impairment in the catheterized RA.

Although the critical role of the endothelium in the regulation of vascular tone has been studied previously, such information is from either animal models [15, 16] or patients undergoing transradial catheterization for coronary angiography and/or angioplasty [46-49]. In the latter case, endothelial dysfunction is likely to be apparent prior to catheterization [385]. To our knowledge, this is the first study providing direct evidence of impaired shear-mediated dilation following catheterization in young healthy trained individuals with an

optimally functioning endothelial layer *a priori*. Interestingly, we observed completely abolished FMD in 1/5th of participants following catheterization. It is worth noting that when comparing the effect of catheterized arm with the changes in the control arm, only 10 out of 23 participants do not show large decrease in FMD post-catheterization. This observation demonstrated the importance of including a control measurement in studies assessing FMD. The variation in response (completely abolished or only impaired FMD) may be due to the different degrees of impact of the catheterization in endothelial function. The external diameter of the catheter sheaths used in this study were 0.9 or 1.2 mm and mean pre-procedure internal radial artery diameter was ~2.3 mm. J-shaped guidewires were also used, with the aim of harvesting ECs. Given the fragile nature of the endothelial monolayer it is likely that the endothelium was impacted in participants with abolished FMD. Therefore, our data from young trained individuals support animal models of endothelial denudation, where balloon inflation or catheterization leads to abolished FMD [15, 16], suggesting the presence of an intact endothelium is essential for shear-mediated dilation. In contrast, Dawson *et al.* (2010), observed reduced, but not abolished, FMD 24 hours post catheterization, induced with much larger 6F (2.7 mm external diameter) catheters, in CAD patients (n=13) [46]. Recently published data from our group **(Chapter 4)** further supports this finding, showing that FMD is impaired, but not abolished, in CAD patients (n=33) 7 days post-denudation [13]. Of importance, in the current study, J-shaped wires (external diameter 3 mm) were used in addition to catheters (5-7F catheters) [13], which may also explain the potential difference for inducing endothelial dysfunction between the groups in this thesis.

The exaggerated response (abolished or negative FMD) observed in young trained individuals compared to CAD patients raises the possibility that shear-mediated dilation may be regulated by different mechanisms, depending on the characteristics of the participants,

i.e. those with diseased vs. optimally functioning endothelium. Indeed, Dawson *et al.* (2010) suggested that the presence of dilation following endothelial denudation may point to an endothelium-independent mechanism contributing to shear-mediated dilation in CAD patients [46]. To support this hypothesis, a meta-analysis by Green *et al.* (2014) previously stated that the contribution of nitric oxide (NO) to FMD may be smaller in CVD patients, when compared to healthy individuals [346]. Endothelial-derived relaxing factor (EDRF) represented a mechanism of diffusible factors and/or electrical signals that causes hyperpolarization of VSMC and cause dilation, either directly (endothelium-independent) or indirectly thought hyperpolarization of endothelial cells which passively spread to VSMC [391]. EDRF still remained a concept in regulating vascular tone and it has also implied that its contribution on vasodilation may be increased in CVD conditions and thus *a priori* endothelial dysfunction [391]. In addition, other perhaps yet unknown vasodilators may contribute to shear-mediated dilation thought endothelium-independent pathways and possibly exceed NO-contribution in cases with apparent endothelial dysfunction (reduced NO-bioavailability). Future studies should investigate the contribution of endothelium-dependent and independent vasodilators in different populations, to elucidate the mechanisms of shear-mediated dilation.

Baseline diameter was higher in the catheterized artery 7 days post-catheterization compared to pre-catheterization, while no change was observed in the control artery, supporting the suggestion that catheter impacts were unilateral in nature. Interestingly, we found that peak diameter during FMD, in addition to baseline diameter, was elevated following catheterization. Earlier studies investigating the influence of catheterization in CAD patients have only reported increased baseline diameter, with no effect on peak diameter

[46-49]. Consequently, it is unclear whether elevated peak diameter occurs in all populations or is present only in young trained individuals, as an advantage of healthier arteries.

In order to isolate the local effect of catheterization in the shear-mediated dilation, we calculated the FMD change in the catheterized artery pre-post catheterization, and subtracted this from the similarly calculated FMD change in the non-catheterized artery. This approach normalises for any systematic variability and allowed us to further explore factors that may contribute to FMD impairment following catheterization. Except for 6 participants whose shear-mediated dilation appeared to not be greatly affected by catheterization, there was a pronounced reduction in FMD response in the catheterized artery following endothelial disruption. Indeed, almost half of participants (9 out of 23) reported a significant reduction in FMD (>5%), after normalising data from the systemic variability. This further supports the localised nature of the impact of catheterization on FMD in young healthy well-trained individuals.

Although smaller RA baseline diameter prior to catheterization has been associated with increased radial artery occlusion risk [392], and a larger magnitude of endothelial dysfunction (i.e. lower radial artery FMD) [393] following PTCA and/or PCI, our data revealed no association between baseline diameter and FMD impairment post-catheterization. In addition, neither catheter size nor artery-to-sheath ratio appeared to result in greater FMD impairment following catheterization. Importantly, in the present study, the baseline arterial diameter (~2.3 mm) was larger than the sheath external diameter (0.9-1.2 mm) in all participants, which could explain the absence of correlations between baseline diameter, catheter size and artery-to-sheath ratio with FMD impairment. Indeed, Saito *et al.* (1999), observed greater flow reduction in the radial artery in patients with artery-to-sheath ratio <1,

compared to those with artery-to-sheath ratio >1 [394]. Similarly, although our findings revealed no correlation between FMD impairment and age, BMI, or fitness level, the lack of association in our study may be explained by the relatively small sample size and narrow range of subjects across these variables.

From a clinical perspective, it has been hypothesised that optimizing the function and size of arteries prior to catheterization may limit the impact of transradial catheterization and improve arterial health post-procedure. As such, preoperative exercise-based rehabilitation has been suggested prior to transradial catheterization [294], due to the well-established benefits of exercise training on arterial function and outward remodelling [25, 37, 143, 163]. Although our current data in fit healthy young subjects indicates that preoperative exercise-based rehabilitation is unlikely to fully negate the impact of catheterization on arterial function, the well-established benefits of exercise training on artery function, structure and health should not be discounted.

This study had a number of limitations. We recruited young healthy males, and given the effect of oestrogen in endothelial function, we could not generalise the results to females. In addition, we did not control for age, BMI, VO_{2peak}, catheter size or artery-to-sheath ratio. However, we performed Pearson correlations between FMD change and the aforementioned factors with no associations. The relatively small sample size and narrow range of subjects across these variables may explain the absence of such correlations. In addition, we did not infuse vasodilators such as nitroglycerine during the catheterization. Given that the use of these vasodilators is consisted a common practise in transradial catheterization in patients in order to increase the arterial diameter, it is possible that the lack of these drugs may affect the vascular outcomes in the present study. Further work

should include vasodilators during catheterization to determine if such FMD abolition in healthy individuals is due to the absence of vasodilators or an exaggerated response in individuals with an intact endothelium. However, artery-to-sheath ratio was always over 1 and neither artery-to-sheath ratio nor baseline diameter appeared to be associated with FMD impairment, therefore it is unclear whether larger arterial size-induced by vasodilators would significantly affect our results of reduced endothelial-dependent dilation 7-days post-catheterization.

This study provides novel information that shear-mediated dilation in young healthy trained individuals is impaired, and in 1/5th of participants abolished, as a result of transradial catheterization. When combined with earlier studies in CAD patients [46-49], where FMD was impaired but not abolished, our data suggests our data suggests the endothelial contribution to FMD may be larger in healthy, well-trained individuals than in subjects with *a priori* endothelial dysfunction. In CAD patients who have *a priori* endothelial dysfunction, further damage/denudation due to catheterisation may have a lesser effect on the FMD response. CAD patients' FMD may be mediated by other mechanisms (i.e. EDRF), apart from NO, which appeared to contribute to a large extent to FMD response of apparently healthy subjects. Another explanation for such exaggerated %FMD post-damage in young healthy compared to CAD patients may be that CAD patients' FMD is typically lower than in healthy individuals due to the presence of endothelial dysfunction, and consequently, it may be reduced to a lesser extent following additional damage induced by catheterization. Neither baseline diameter, age, BMI, fitness level, nor catheter size or artery-to-sheath ratio were associated with FMD reduction in this experiment. Our novel approach to studying arterial responses following catheterization in humans may inform future studies that could

directly compare healthy individuals and clinical populations and elucidate endothelial contribution to changes in vascular function and remodelling *in vivo*.

CHAPTER 7 - Conduit artery endothelial cell PECAM-1 is not phosphorylated by handgrip exercise in young healthy humans.

7.1. ABSTRACT

Although clear evidence demonstrates the fundamental role of shear stress in vascular health, predominantly through the release of nitric oxide (NO), the mechanisms by which endothelial cells (EC)s sense and transduce shear are poorly understood. In cultured ECs tyrosine phosphorylation of PECAM-1 has been shown to activate eNOS in response to shear stress. However, in the skeletal muscle microcirculation PECAM-1 was not activated in response to passive leg movement in humans. Given this contradiction, this study aimed to assess the effect of exercise on conduit arterial PECAM-1 and eNOS activation in humans. Eleven males were randomised to two groups; exercise group (n=6), who performed 30 minutes of handgrip exercise, and a time-control group (n=5). Protein expression of eNOS and PECAM-1, alongside eNOS Ser^{1177} and PECAM-1 Tyr^{713} phosphorylation were assessed in ECs obtained from the radial artery pre and immediately post each intervention. Handgrip exercise resulted in a 5-fold increase in mean shear stress in the exercise group, with no change in the control group (group*time, $P<0.001$). There was a 54% increase in eNOS Ser^{1177} phosphorylation in the exercise group, when compared with control group (group*time, $P=0.016$), whereas no change was reported in PECAM-1 Tyr^{713} phosphorylation in either group (group*time, $P>0.05$). eNOS and PECAM-1 protein content was unchanged in pre- to post-intervention (group*time, $P>0.05$). Our data, suggest that elevations in conduit artery shear stress, mediated by exercise, do not phosphorylate PECAM-1 in ECs of young active males, suggesting PECAM-1 is not involved in the vascular response to prolonged exercise in conduit arteries of young healthy men.

7.2. INTRODUCTION

Endothelial cells (EC)s play a crucial role in controlling vascular tone and homeostasis [54] and are responsible for the expression of pro- and anti-atherogenetic genes [55]. Many of these important effects are mediated by the release of nitric oxide (NO) in response to hemodynamic stimuli exerted on the luminal surface of ECs by the frictional force of the flowing blood on the ECs (shear stress) [395, 396].

Although there is clear evidence demonstrating the fundamental role of shear stress in vascular health [397], the mechanisms through which ECs sense and transduce shear are poorly understood [277]. It has been proposed that changes in fluid shear stress are sensed from the apical surface of the endothelial cell, in particularly at cell–cell and cell–matrix junctions [277], where this mechanical tension is transduced to several biochemical signals, including NO production. Studies on cultured ECs have demonstrated that shear stress is sensed by a mechanosensory complex, including platelet endothelial cell adhesion molecule 1 (PECAM-1) [397]. In culture elevations in shear stress lead to Tyrosine phosphorylation of PECAM-1 and subsequent downstream activation of endothelial nitric oxide synthase (eNOS) [277], the enzyme responsible for NO production. However, *in vivo* studies investigating the activation of PECAM-1 in the skeletal muscle microvasculature have not shown increased PECAM-1 phosphorylation in response to increased shear stress, induced by passive leg movement [284]. As such, the role of PECAM-1 in sensing shear stress and subsequent downstream activation of eNOS is unclear in humans.

Therefore, the aim of this study was to assess the effect of exercise on arterial PECAM-1 and eNOS activation in humans. We hypothesised that acute exercise would phosphorylate both PECAM-1 and eNOS in arterial endothelial cells of healthy young males.

7.3. METHODS

Subjects

Eleven healthy, active (≥150 minutes of moderate-intensity or ≥75 minutes of vigorous-intensity exercise per week), young (<40 years) males volunteered to participate in this study (Table 7.1). Participants were randomly assigned to either exercise (n=6) or time-matched control (n=5) groups. Participants were non-smokers and free of diagnosed cardiovascular disease (CVD), or CVD risk factors such as diabetes, hypertension or hypercholesterolemia. Informed consent was gained from all participants prior to the experimental procedures. The study conformed to the Declaration of Helsinki, and ethical approval was obtained from the Liverpool North-West NHS Research Ethics Committee (REC 18/NW/0428).

Pre-Experimental Visit

At least 72 hours prior to the experimental visit participants attended the laboratory to complete an incremental exercise test to exhaustion on an electromagnetically braked cycle ergometer (Corival, Lode, Groningen, Netherlands) to determine peak oxygen consumption (VO_{2peak}), as descripted in **Chapter 3**. Participants' handgrip strength, as determined by a maximum voluntary contraction test (MVC) (Takei 5420 Grip-D Digital Hand Grip Dynamometer, Japan), was also measured as descripted in **Chapter 3**.

Experimental Trial

Participants attended the cardiovascular lab at Liverpool John Moores University, having fasted overnight and abstained from caffeine, alcohol and vigorous exercise the day before. Experiments were commenced between 7am and 1pm, in a quiet temperature-controlled room.

Upon arrival, participants rested in the supine position for at least 10 minutes to ensure all hemodynamic variables were stabilised. Arterial catheterization and EC collection were then performed in the radial artery, as descripted in **Chapter 3**. Participants subsequently undertook either 30 minutes of forearm exercise or remained rested for 30 minutes (control). Hemodynamic and blood flow variables were collected before and during each intervention. EC collection was repeated immediately after the forearm exercise protocol or control period.

Handgrip exercise protocol and radial artery blood flow assessment

Participants in the exercise group performed 30 minutes of continuous rhythmic handgrip exercise in the catheterized arm at 15% MVC. Blood pressure and heart rate (GE Pro 300V2, Dinamap, Tampa, FL, USA) were measured prior to and every 10 minutes during the exercise. A 12-MHz multi-frequency linear array probe, attached to high-resolution ultrasound (T3000; Terason, Burlington, MA, USA), was used to image diameter and velocity of the experimental radial artery (15-18 cm proximal to the wrist) prior to and every 10 minutes during the exercise protocol, as descripted in **Chapter 3**. Rating of perceived exertion (RPE) on a 1-10 scale (1: no effort to 10: maximal effort) was taken at the end of exercise. Blood pressure, heart rate and radial artery vascular variables were also assessed in the time-control group at the same time-points. The same ultrasound and sonographer were used between and within participants.

EC protein expression via immunofluorescence

Details of the procedures used have been descripted in **Chapter 3**. Briefly, cells were recovered by centrifugation. Collected cells were fixed with 3.7% formaldehyde and plated onto glass slides and then frozen at -80°C until analysis. See **Chapter 3** for further information about staining protocol, image capture and image analysis. Importantly for this Chapter, slides were incubated with primary antibodies against eNOS (610297, BD, USA), peNOS Ser^{1177} (07-428-I, Merck), PECAM-1 (abc24590, Abcam, UK) and pPECAM-1 Tyr^{713} (BS4666, Bio World, USA). Slides within participants were stained and imaged in the same batch using the same microscope and analysis settings, and relative difference between pre- and post-intervention slides was assessed. A single blinded technician completed all imaging and analysis. eNOS and peNOS Ser^{1177}, and PECAM-1 and pPECAM-1 Tyr^{713} were stained on the same slide, as such, it was possible to establish eNOS Ser^{1177}/eNOS and PECAM-1 Tyr^{713}/PECAM-1 ratio on an individual cell basis, as the same cytosolic outline could be placed over both content and phospho images.

Data analysis and statistics

Custom-designed edge-detection and wall-tracking software was used to analyse changes in vascular variables in the exercise and control groups, in order to minimise investigator bias [342, 348]. See **Chapter 3** for more details. Changes in vascular variables (diameter, blood velocity, blood flow, shear rate) were calculated in averages of usually 1-minute recordings, before, and every 10 minutes during exercise, as descripted in **Chapter 3.**

All statistical analyses were performed using IBM SPSS statistics for Windows, version 25.0. Armonk, NY: IBM Corp. Radial artery hemodynamic and shear variables as well as EC

protein expression were compared via two-way repeated-measures ANOVA to detect differences within and between conditions. Pairwise comparisons were performed when significant main or interaction effects were detected, using Fisher's least significant difference (LSD) test. Data are presented as mean±SD and alpha significance was set at P≤0.05.

7.4. RESULTS

There were no differences in age, VO_{2peak} and handgrip strength between groups. However, body mass index (BMI) was significantly higher in the exercise group when compared to control. Values are presented in Table 7.1.

Table 7.1. Summary of participants' characteristics.

	Exercise (n=6)	Control (n=5)	P value
Age (years)	27.3±6.5	24.6±5.2	0.466
BMI (m/kg^2)	26.0±1.5	22.2±0.8	0.001
VO_{2peak} (ml/kg/min)	51.2±12.1	53.3±13.0	0.800
MVC (kg)	43.2±4.4	38.1±4.1	0.429

BMI: body mass index; $VO_{2peak:}$ peak oxygen consumption; MVC: maximal voluntary contraction.

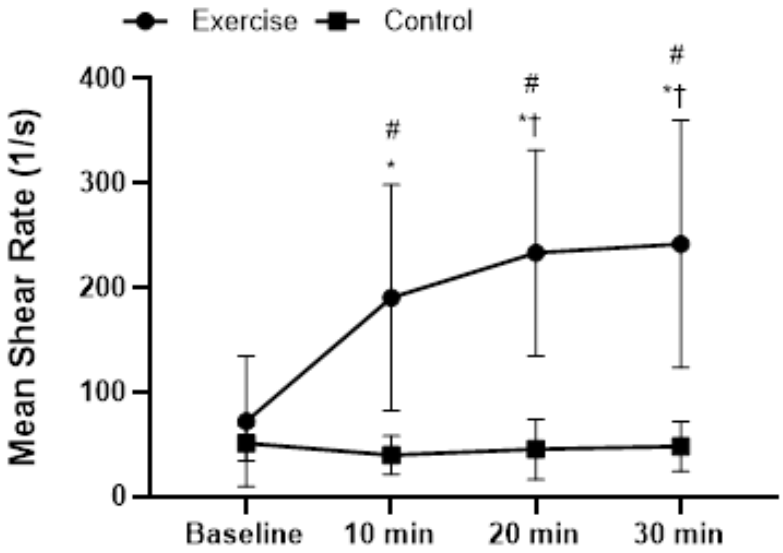
Radial artery hemodynamics

There was a significant interaction effect (group*time) for radial artery mean shear rate (P<0.001), blood velocity (P<0.001), blood flow (P<0.001), anterograde blood flow (P<0.001) and anterograde shear rate (P<0.001). Pairwise comparisons demonstrated that

mean shear rate during handgrip exercise was increased from baseline throughout the exercise protocol (P≤0.001) (Figure 7.1), increasing from 10 to 20 (P=0.017) and 30 minutes (P=0.044). In contrast, mean shear rate was unchanged from baseline in the control group (P>0.1). Baseline mean shear rate was not different between the groups (P>0.1), but was significantly increased in the exercise group at 10 minutes (P=0.025), 20 minutes (P=0.001) and 30 minutes (P=0.012) compared to control. Blood velocity, blood flow, anterograde blood flow and anterograde shear rate followed the same pattern as mean shear rate (Table 7.2). In contrast, retrograde blood flow (P=0.022) and retrograde shear rate (P=0.035) displayed a main effect of time, with no main effect of group (retrograde blood flow: P=0.051, retrograde shear rate: P=0.315) or interaction (retrograde blood flow: P=0.096, retrograde shear rate: P=0.095) observed. Finally, radial artery diameter was smaller in the control group compared to exercise group (2.4 mm vs. 2.9 mm; main effect of group P=0.015). There was a main effect of time (P=0.046), however there was no interaction effect (P=0.205), suggesting that arterial diameter in the control group was typically smaller from the baseline and during the intervention. Results including pairwise comparisons are presented in Table 7.2.

Heart rate did not change in either group during the trial (group*time, P=0.877). However, a significant interaction effect (group*time) was observed for mean arterial pressure (P<0.001) (Table 7.2). Mean arterial pressure was increased during the handgrip exercise protocol at 20 minutes (P=0.009) and 30 minutes (P=0.005) compared to baseline, whereas no change was observed at 10 minutes compared to baseline (P=0.140). Conversely, mean arterial pressure in the control group was significantly reduced at 30 minutes compared to baseline (P=0.031), while no change was reported at 10 minutes (P=0.057) and 20 minutes

(P=0.132), compared to baseline. In general, mean arterial pressure was higher in the exercise group than control (main group effect P=0.006).



<u>Figure 7.1.</u> Mean shear rate in the exercise (n=6) and control groups (n=5) prior to intervention (Baseline) and at 10, 20 and 30 minutes.

Results are presented as mean±SD, $P<0.05$. *Significantly different from Baseline, †Significantly different from 10 minutes, #Significantly different from control group.

Table 7.2. Radial artery hemodynamics in the exercise (n=6) and control groups (n=5), prior to intervention (Baseline) and at 10, 20 and 30 minutes.

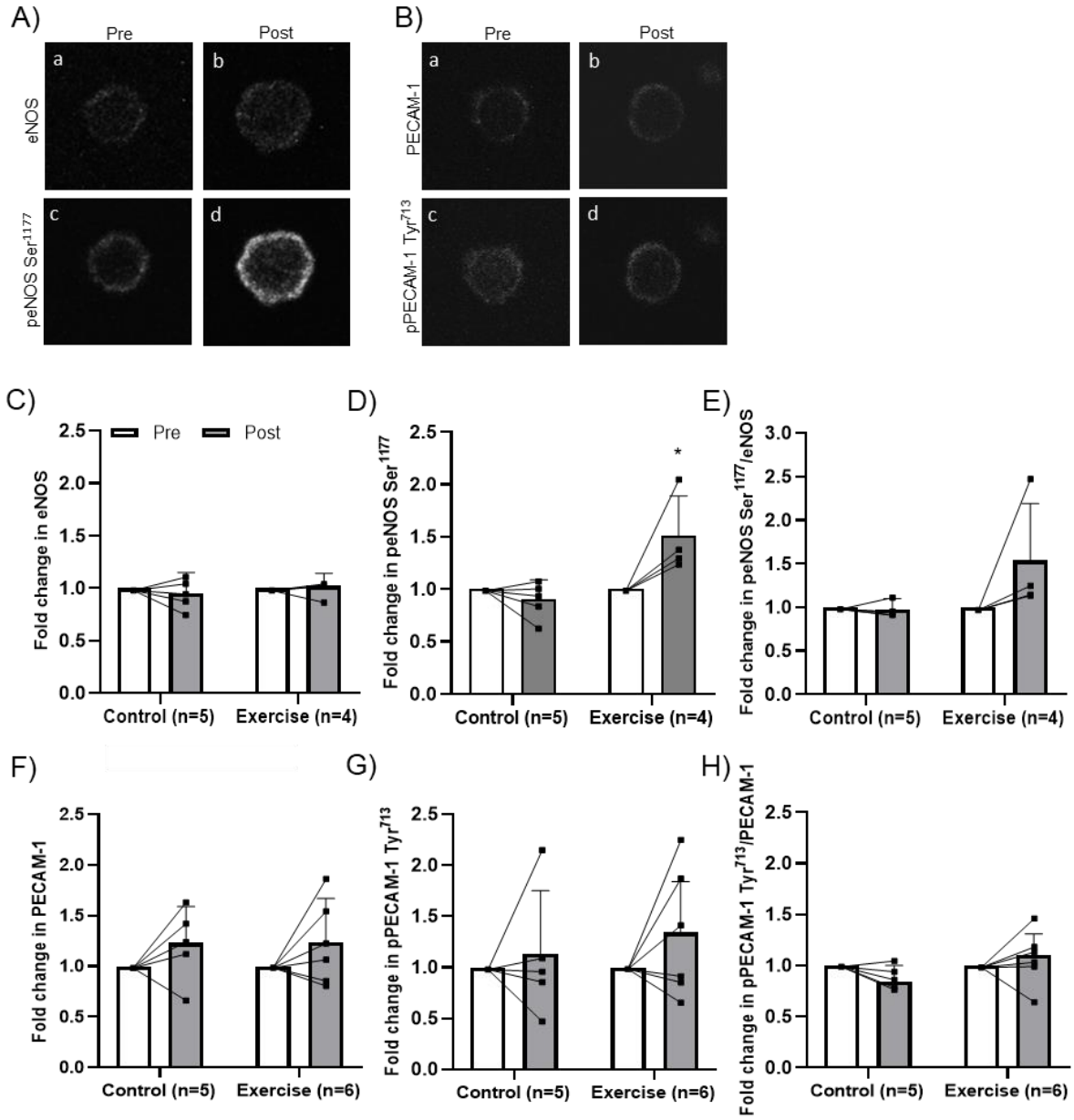
		Baseline	10 minutes	20 minutes	30 minutes
Diameter (mm) [#]	Exercise	2.77±0.4	2.99±0.4	2.93±0.4	3.02±0.4
	Control	2.33±0.2	2.39±0.3	2.45±0.1	2.37±0.2
Blood velocity (cm/min) [#‡]	Exercise	4.84±4.0	13.74±7.5[*]	16.97±6.9[*†]	17.88±8.5[*†]
	Control	3.05±1.3	2.41±1.0	2.81±1.5	2.99±1.6
Blood flow (ml/min) [#‡]	Exercise	16.56±12.7	54.73±27.3[*]	68.17±26.6[*†]	72.25±32.7[*†]
	Control	8.47±4.6	7.02±3.5	8.54±4.5	8.83±5.2
Anterograde flow (ml/min) [#‡]	Exercise	18.23±12.0	66.15±26.2[*]	82.32±21.9[*†]	84.50±30.0[*†]
	Control	11.34±6.3	10.94±5.1	12.85±6.1	12.57±7.2
Retrograde flow (ml/min)	Exercise	-1.72±2.3	-11.42±4.9	-14.15±10.7	-10.24±6.7
	Control	-2.31±2.1	-3.67±2.1	-4.44±2.0	-4.40±2.3
Anterograde shear rate (1/s) [#‡]	Exercise	79.32±58.5	232.55±117.7[*]	283.51±84.5[*†]	277.70±121.1[*†]
	Control	68.39±23.1	61.23±21.8	67.29±41.2	68.71±31.4
Retrograde shear rate (1/s)	Exercise	-6.93±8.6	-42.05±31.6	-50.11±37.2	-35.99±22.5
	Control	-17.23±9.6	-21.55±7.1	-21.95±13.8	-20.50±8.0
Heart rate (beats per min)	Exercise	65±15	65±13	67±14	66±12
	Control	59±17	61±11	63±8	63±10
Mean arterial pressure (mmHg)	Exercise	101±8	105±11	109±8*	108±14*
	Control	91±6	86±6	88±6	87±7*

Results are presented as mean±SD, $P<0.05$. [#]Main group effect, [‡]Main interaction effect (group*time), [*]Significantly different from Baseline, [†]Significantly different from 10 minutes.

EC protein expression and phosphorylation

eNOS content was unchanged in either group following the intervention (group*time, P=0.557) (Figure 7.2A). In contrast, a significant interaction effect was observed in eNOS Ser^{1177} phosphorylation (P=0.016). Pairwise comparisons show higher eNOS Ser^{1177} phosphorylation in the exercise group (~54% increase, P=0.009), whereas no change was observed in the control group (P=0.499) (Figure 7.2B) In addition, eNOS Ser^{1177} phosphorylation was higher in the exercise group than in the control group following intervention (P=0.016). When normalised to eNOS content eNOS Ser^{1177} phosphorylation (peNOS Ser^{1177}/eNOS) was unchanged in both groups following intervention (group*time P=0.1) (Figure 7.2C).

PECAM-1 was unchanged in either group following the intervention (group*time, P=0.968) (Figure 7.2D). PECAM-1 Tyr^{713} phosphorylation was unchanged in either group following intervention (group*time, P=0.579) (Figure 7.2E). Equally, when normalised to PECAM-1 content PECAM-1 Tyr^{713} phosphorylation (pPECAM-1 Tyr^{713}/PECAM-1), was unchanged in either group post intervention (group*time, P=0.109) (Figure 7.2F).



<u>Figure 7.2.</u> eNOS, eNOS Ser^{1177} phosphorylation (peNOS), PECAM-1 and PECAM-1 Tyr^{713} phosphorylation (pPECAM-1) in the exercise and control groups pre- and post-intervention.

A) Representative confocal microscopy images of radial artery endothelial cells (EC)s stained for eNOS (a, b) and peNOS Ser^{1177} (c, d) pre (a, c) and post (b, d) handgrip exercise. B) Representative confocal microscopy images of radial artery EC stained for PECAM-1 (a, b) and pPECAM-1 Tyr^{713} (c, d) pre (a, c) and post (b, d) handgrip exercise. Mean

fluorescence intensity of eNOS (C), peNOS Ser^{1177} (D), PECAM-1 (F) and pPECAM-1 Tyr^{713} (G) is summarised. E) displays peNOS Ser^{1177}/eNOS ratio and H) displays pPECAM-1 Tyr^{713}/PECAM-1 ratio. The mean fluorescence intensity pre intervention was assigned a value of 1, and the relative intensity post intervention was calculated. Results are mean±SD, *Significantly different from Pre (P<0.05).

7.5 DISCUSSION

This study demonstrated that elevated shear rate induced by a single bout of handgrip exercise stimulates eNOS Ser^{1177} phosphorylation, but does not lead to Tyr^{713} phosphorylation of PECAM-1 in ECs obtained from the radial artery of young active males. Therefore, this study provides further evidence that PECAM-1 is not activated by prolonged elevations in shear stress in humans.

The major outcome of the present study was that PECAM-1 Tyr^{713} phosphorylation was not elevated in human ECs following a 5-fold increase in shear stress induced by exercise. This is the first study to investigate conduit artery endothelial PECAM-1 phosphorylation in response to exercise. However, this observation is in line with a recent study reporting no change in PECAM-1 Tyr^{713} phosphorylation in the skeletal muscle microcirculation, following hyperaemia generated by passive leg movement [284]. Together these studies suggest that endothelial PECAM-1 is not activated in response to prolonged (20-30 minutes) elevations in shear stress, in humans. This is in contrast to observation from cultured ECs, where the application of fluid shear stress (12 dynes cm–2) elicited marked tyrosine phosphorylation of PECAM-1 [278]. Importantly, decreasing EC expression of PECAM-1, using siRNA oligonucleotides or ECs isolated from PECAM-1 knockout mice, blunted eNOS Ser^{1177}

phosphorylation and attenuated cyclic GMP levels in response to shear stress [278]. Collectively, the aforementioned studies suggest an inconsistency between *in vitro* and *in vivo* human responses, which raise questions about the role of PECAM-1 in shear stress mediated NO production in humans. The discrepancy between *in vivo* and *in vitro* models may be caused by differences in the magnitude, exposure time and the pattern of shear stimulus between cultured ECs and ECs in the *in vivo* setting [281].

Interestingly, using muscle arterioles isolated from PECAM-1 knockout mice, Bagi *et al.* [398] observed that the initial dilation of vessels (up to 120 seconds) in response to shear stress was impaired compared to wild type animals, but the second phase of dilation, when shear stress was steady, was similar in the 2 groups. This suggests that PECAM-1 is involved in the initial response to shear stress, whereas other mechanosensory pathways may be required to respond to steady shear stress. In the current study and that of Gliemann *et al.* [284], change in PECAM-1 Tyr^{713} phosphorylation was investigated following a sustained continuous increase in shear stress (30 minutes of continuous handgrip exercise or 20 minutes of passive leg movement [284]), therefore it is possible that any change in PECAM-1 Tyr^{713} phosphorylation was missed. As such, future human studies should examine PECAM-1 phosphorylation status at different stages of shear stimulus (initial vs prolonged).

Our data demonstrates that a single bout of handgrip exercise resulted in a ~54% increase in eNOS phosphorylation at Ser^{1177}, with no effect on eNOS content in human ECs. When normalized to eNOS content, eNOS Ser^{1177} phosphorylation was no longer significantly increased in the exercise group compared to control (P=0.1). However, we believed that this may be explained by the relatively small sample size. This finding of elevated eNOS Ser^{1177}

phosphorylation post exercise agrees with previous work showing a similar increase in eNOS phosphorylation (57%) following 2 hours of handgrip exercise in human ECs collected from the brachial artery [66]. Park *et al.* [399] have also show similar outcomes in response to 1 hour of handgrip exercise in ECs obtained from the radial artery, but the baseline cell collection occurred during cuff occlusion (no blood flow and therefore shear stress). As such, the current study adds physiological relevance as eNOS Ser^{1177} phosphorylation was shown to be elevated when shear stress was increased from resting values.

To conclude, this is the first study to directly assess changes in PECAM-1 activation in response to exercise induced increases in shear stress in conduit arteries of humans. We found that although 30 minutes of handgrip exercise resulted in a 5-fold increase in radial shear stress, and eNOS phosphorylation at Ser^{1177}, PECAM-1 Tyr^{713} phosphorylation was not increased. As such, this study suggests that PECAM-1 activation is not involved in the vascular response to prolonged elevations in shear stress. Given previous observations that PECAM-1 is involved at the onset of shear elevations, future studies should investigate PECAM-1 phosphorylation in response to rapid increases in shear stress.

CHAPTER 8 - General Discussion

8.1. Thesis overview

Cardiovascular disease (CVD) is the leading cause of global mortality, associated with ~17.8 deaths in 2017 [400], while this number is predicted to rise to ~23.6 million by the year 2030 [29]. Although the exact mechanisms leading to CVD vary depending on the disease in question, the progression of atherosclerosis, an inflammatory process resulting in plaque development and thickening of the arterial wall [2], is an underlying aetiological factor for most CVDs.

The presence of plaques, associated with stenosis, in coronary arteries is known as coronary artery disease (CAD). CAD is the most common cause of death from CVD [21, 51]. Diagnosis and treatment of obstructed coronaries often involves procedures requiring catheterization, such as percutaneous transluminal coronary angiography (PTCA) and/or percutaneous coronary intervention (PCI; angioplasty). Although these procedures represent an evolution in the management of CAD, they are likely to mechanically damage the endothelial layer, leading to arterial injury and vascular dysfunction [13]. Determining the extent of catheterization induced injury, as well as the time-course of endothelial recovery from catheterization, are important for the management of CAD, as strategies could be applied to minimize the extent of arterial injury and/or facilitate recovery. For instance, given the well-established benefits of exercise on endothelial function in CAD patients [27, 37], it might be presumed that exercise-based cardiac rehabilitation could improve endothelial dysfunction induced by catheterization. The predominant mechanism thought which exercise benefits endothelial function and consequently CVD, is through an increase in shear stress, the tangential force of the flowing blood on the endothelial surface of blood vessels [25]. However, there is uncertainty regarding the effects of acute exercise on the vasculature following catheterization. Importantly, one study observed a ‘paradoxical’

vasoconstriction of arteries in response to exercise following catheterization in dogs [15]. If such responses are also apparent in humans, there may be a basis to suggest delaying cardiac rehabilitation post-procedure. Assessment of flow mediated dilation (FMD) provides useful information about arterial dysfunction and recovery, and therefore the safest time to embark upon exercise rehabilitation after catheterization. However, there is currently no data on the response of human arteries to exercise following catheterization-induced endothelial damage. Given the complex mechanisms by which exercise regulates blood flow [50], the vascular response of damaged arteries to FMD-induced shear stress may be different from the arterial response to exercise.

This PhD thesis had three general objectives, which have been addressed in four experimental Chapters:

1. Examined the impact of endothelial disruption and/or denudation following catheterization on FMD and arterial response to exercise in CAD patients **(Chapter 4).** Impairments in FMD following catheterization were also examined in young well-trained males with *a priori* healthy arteries **(Chapter 6).**
2. **Chapter 5** explored the relationship between FMD and the arterial response to exercise. I assessed the protein content of eNOS, its phosphorylation at Ser^{1177} (peNOS Ser^{1177}) and other specific atherogenic-modulating proteins related to oxidative stress, inflammation, vasoconstriction and senescence pathways in endothelial cells (EC)s obtained from radial arteries of CAD patients. This provided insights into the molecular events underlying endothelial dysfunction in CAD.

3. **Chapter 7** explored the molecular mechanisms responsible for endothelial nitric oxide (NO) production during exercise in young well-trained males. In particular, I evaluated the role of PECAM-1 in sensing shear stress and the subsequent downstream activation of eNOS. This investigation provided novel direct evidence pertaining to how elevated shear stress induced by exercise benefits endothelial function.

The key outcomes of the present PhD work as well as some directions for further studies are discussed below.

8.2. Key findings

8.2.1. Impact of catheterization-induced damage on FMD

Impaired dilation in response to elevated shear stress has been shown in arteries following endothelial damage and/or denudation. This was first observed in animals [15, 16] and later in CAD patients [46, 47, 49, 163, 355] following catheterization. However, evidence in CAD patients was limited to reduced shear-mediated dilation within 24 hours of catheterization, which recovered ~3 months post procedure. Consequently, there was a gap in the literature regarding the short-term effects of catheterization in human arteries [13]. **Chapters 4 and 6** evaluated the effects of endothelial disruption induced by catheterization 1 week post-catheterization in CAD patients (**Chapter 4**) and young well-trained males (**Chapter 6**). Both studies observed impaired FMD in the catheterized artery 1 week after catheterization, whereas no change was reported in the contralateral artery, suggesting the effects of catheterization are local, not systemic. Importantly, in CAD patients **(Chapter 4)** FMD post-catheterization was reduced, but the response was not completely abolished in any of the participants (Figure 4.2A) In contrast, in approximately 1/5th of young well-trained

participants FMD was abolished following catheterization **(Chapter 6)**. This exaggerated response in young trained individuals, compared to CAD patients, raises the possibility that shear-mediated dilation is regulated by different mechanisms, depending on the characteristics of the participants, diseased vs optimally functioning endothelium. Previously, Dawson *et al.* [46] suggested that the presence of dilation following endothelial denudation in CAD patients may point to an endothelium-independent mechanism contributing to shear-mediated dilation in these patients. Indeed, Green *et al.* [346], suggested that the contribution of nitric oxide (NO) to FMD may be smaller in CVD patients than healthy individuals. These studies support the hypothesis that other mechanisms, perhaps endothelium-independent or at least not NO-mediated, may drive the FMD response in cases where endothelial dysfunction is apparent. Endothelial-derived relaxing factor (EDRF) may represent a mechanism (diffusible factors, perhaps yet unknown, or electrical signals) that causes hyperpolarization of VSMC, either directly or indirectly thought hyperpolarization of endothelial cells which passively spread to the VSMC [391]. EDRF remains a concept in regulating vascular tone and it has also been suggested that its contribution to vasodilation may be increased in subjects with CVD conditions [391].

8.2.2. Arterial response to acute exercise pre- and post-catheterization.

A previous study in dogs reported ‘paradoxical’ vasoconstriction in response to exercise following endothelial denudation [10]. This has raised concerns regarding the early application of exercise-based rehabilitation following catheterization. **Chapter 4** presents the first evidence in CAD patients that catheterization does *not* lead to impaired exercise-mediated vasodilation, 7 days after the procedure. This contrasting data in humans suggests it may be safe to undertake exercise-based rehabilitation soon after PTCA and/or PCI.

Interestingly, **Chapter 4** observed that vasodilation in response to exercise was preserved, but FMD was impaired 1 week following catheterization. This suggests that the impact of catheterization on functional arterial responses in humans may be stimulus specific. A potential explanation for the contrasting responses to exercise and FMD could be due to the fact that FMD is an endothelium-dependent response mediated primarily through endothelial NO production [346], whereas the arterial response to exercise is more complex, involving a number of mechanisms and vasoactive compounds with many interactions [358, 359]. Exercise causes an increase in blood pressure, release of metabolites and gradual increase in shear stress; all of which can regulate blood flow and consequently vasodilation [25, 50]. Thus, we suggest that arterial responses to exercise following catheterization may be preserved by other compensatory "redundant" pathways. In addition, we believe that our findings of stimulus specific arterial outcomes highlight the importance of applying multiple techniques to evaluate arterial function in humans. To further support the above, **Chapter 5** indicated that FMD was not correlated with handgrip exercise-induced vasodilation in CAD patients, which reinforces the suggestion that different mechanisms underlie the two responses, at least in CAD. Consequently, we suggest that assessing vascular responses to exercise could provide an ecologically valid assessment to complement FMD in future studies, particularly as the exercise response is the most relevant test to provide insights into exercise-based rehabilitation in CAD patients following catheterization. However, there are no data associating arterial response to exercise with cardiovascular risk, therefore it is currently unknown whether there is a prognostic value for this test. We believe that this may be an interesting and relevant question for future large-scale studies.

8.2.3. Radial artery vasodilation following handgrip exercise in CAD patients and young heathy males

Chapter 5 demonstrated that handgrip exercise resulted in radial artery vasodilation in CAD patients. However, radial artery diameter did not increase in a stepwise manner to incremental handgrip exercise at 5%, 10% and 15% MVC. To our knowledge, this is the first study providing direct data that the radial artery of CAD patients (n=54) is able to dilate in response to exercise. However, our pilot study (12 young healthy males) showed that arterial diameter at 15% MVC was significantly increased when compared to 5% and 10% MVC, suggesting the possibility of a stepwise increase in radial artery diameter in response to incremental handgrip exercise in young healthy individuals **(Chapter 3)**. Perhaps the workload increase from 5% to 10% MVC was not adequate to stimulate further dilation. Given the relatively small sample in this pilot study (n=12), further studies with larger sample size and perhaps higher HE intensities' required to clarify the stepwise increase in diameter in young healthy subjects. However, the fact that the exact same HE protocol was able to cause changes in arterial diameter in 12 young males may suggest that our CAD data of 54 patients is unlikely to be underpowered. To further investigate the difference among young healthy and CAD patients, we performed a 3-way ANOVA, between 54 CAD patients **(Chapter 5)** and 12 young healthy participants **(Chapter 3).** Briefly, there was a main group effect ($P=0.002$), main intensity effect ($P<0.0001$) and main interaction effect (exercise intensity*group) ($P<0.0001$). Arterial diameter was overall higher in CAD group compared to young subjects. In regards to HE response, there was a stepwise increase in diameter (expect from 5% to 10% MVC) in young healthy subjects, whereas in CAD patients HE resulted in increased diameter from baseline, independently of the exercise intensity. Although there was a great difference in sample size between the groups (54 vs. 12), which potentially affect the outcomes, the exercise protocol was exactly the same between the two

groups (3 X 3-minute bouts of 5%, 10% and 15% MVC, with 1-minute rest between bouts), suggesting that this difference may reflect impaired arterial response to exercise in CAD patients. Further work is needed to test if this is seen in a larger cohort.

8.2.4. Endothelium-dependent dilation is related to eNOS Ser^{1177} phosphorylation and senescence markers (p21, p16). The role of peNOS Ser^{1177}.

Chapter 5 demonstrated that endothelium-dependent dilation was positively related to eNOS Ser^{1177} phosphorylation in arterial endothelial cells of CAD patients. This finding is in contrast to some observations in healthy ageing, where reductions in endothelium-dependent vasodilation were not associated with eNOS Ser^{1177} phosphorylation [76]. Given that exercise training is likely to result in greater FMD improvements in subjects with *priori* endothelial dysfunction [401, 402], perhaps eNOS Ser^{1177} phosphorylation status may represent a potential mechanism. Indeed, 8 weeks of exercise training resulted in elevated eNOS Ser^{1177} phosphorylation in arteries of CAD patients [147]. However, to the best of our knowledge, there is no information about the effects of exercise training on eNOS Ser^{1177} phosphorylation in conduit arteries of healthy subjects, therefore the above remains a speculation and further studies in different cohorts should be conducted. In addition, we did not find a correlation between endothelium-dependent dilation and markers of oxidative stress (nitrotyrosine or protein content of NOX2). This also contrasts with observations in healthy ageing, where endothelium-dependent dilation is negatively associated with oxidative stress markers [88]. Taken together, these findings suggest a difference in the regulation of endothelium-dependent dilation between patients with established CAD and apparently healthy humans. We suggest that, in patients with established CAD, further elevations in oxidative stress do not contribute to progressive declines in endothelium-

dependent vasodilation, with maintaining activation of eNOS through Ser^{1177} phosphorylation becoming increasingly important to EC NO bioavailability. However, given that our study **(Chapter 5)** only assess the EC expression of oxidative proteins and FMD in patients with established CAD (obstructive coronaries vs. non-obstructive coronaries), further studies including young healthy individuals and age-matched controls should be conducted to clarify the above hypothesis.

Chapter 5 also suggested a positive association between the protein content of senescence markers (p21, p16) and endothelium-dependent dilation. These associations were unexpected and, due to the lack of human studies investigating the role of senescence in the development of CVD, somewhat difficult to explain. Indeed, although cellular senescence has been implicated in the development and progression of CVD [8], there are a lack of studies providing direct evidence of elevated senescence markers in the endothelium of CVD patients, compared to healthy individuals, and how increased senescence may impact endothelial function. To date, only one study has investigated the potential link between senescence and endothelium-dependent dilation, demonstrating that age-associated reductions in FMD were negatively associated with endothelial expression of p21, p53 and p16 [126]. Interestingly, studies in murine models of high-fat diet induced atherogenesis have shown that knockout of p53 [377] and p21 [378] resulted in more atherosclerotic lesions. As such, the role of senescence within the vasculature may depend on the stage of atherosclerotic progression [133] [135] with senescence initially playing a protective role. Future studies should test the causal contribution of senescence to endothelial dys/function and how this may be altered in health and at different stages of CAD. In particular, animal studies using the gene knock-out techniques would be able to first study the causal link between senescence and endothelial function and secondly to

investigate whether senescence had a different role across the lifespan and in diseases. For instance, although it is known that senescence factors are generally increased by age, perhaps in certain diseases such as cancer or CVD, senescence may protect and/or slow down the tissue dysfunction due to the activation of apoptosis and/or permanent cell arrest pathways. In addition to animal studies, large-scale human studies should be followed to investigate whether there is a difference in senescence among different stages of CAD or different risk factors for CVD and/or with age-matched controls.

8.2.5. PECAM-1 is not phosphorylated following acute exercise

Chapter 7 demonstrated that elevated shear rate, induced by an acute bout of handgrip exercise, stimulates eNOS Ser^{1177} phosphorylation, but does not lead to PECAM-1 Tyr^{713} phosphorylation in endothelial cells (EC)s obtained from young healthy active males. To our knowledge, this is the first study to investigate PECAM-1 phosphorylation in response to acute exercise, and its association with eNOS activation. However, the data are similar to a recent study where 20 minutes of passive leg movement also did not result in PECAM-1 Tyr^{713} phosphorylation in the skeletal muscle microcirculation [281]. In contrast, PECAM-1 activation in response to increased shear stress has been observed in cultured ECs [278]. In addition, Fleming *et al.* [278] suggested that PECAM-1 phosphorylation modulated eNOS activity in response to shear stress. However, when the findings of **Chapter 7** are taken together with those of Gliemann *et al.* [281], the role of PECAM-1 as a mechanosensor in human ECs becomes unclear. Interestingly, in PECAM-1 knockout mice only the initial phase of vasodilation (within 120 seconds) in response to shear stress was shown to be impaired, compared to wild type animals, suggesting PECAM-1 plays an important early role in the endothelial shear stress response [398]. As such, it is possible that we missed any change in PECAM-1 Tyr^{713} phosphorylation due to ECs being collected following 30 minutes

of steady state exercise. Therefore, I believe future studies are required to examine PECAM-1 Tyr^{713} phosphorylation at different stages of shear stimulus (onset, middle, end), clarifying whether PECAM-1 responds to elevated shear stress in humans or not.

8.3. Directions for future research

8.3.1. Time-course of endothelial function recovery following catheterization and the potential role of exercise training.

Chapter 4 showed impaired FMD in CAD patients 1 week following PTCA and/or PCI. Together with previous research on the time-course of endothelial recovery from catheterization, it appears that endothelial function following catheterization recovers at some point between 1 and 12 weeks. Although our research has added important information to this timeline, further studies are required to understand when in this window recovery takes place. This information is important for a clinical prospective in CAD, as reduced FMD (1% decrease) has been associated with a significantly increased risk (8-22%) of future CVD events [9, 10]. To further support the above, FMD post-PCI appeared to predict late restenosis or revascularization rates induced by coronary stenting [189, 192, 193]. It is worth noting that the above studies associated brachial FMD in a large-scale population of CAD patients post femoral catheterization reflecting systemic endothelial function, suggesting that endothelial function can be used as an independent predictor for cardiac events or in-stent restenosis and revascularization. Although the majority of studies evaluating FMD post transradial catheterization, did not report systemic endothelial dysfunction but only local [13, 46, 49, 163], it is likely that catheterized coronaries may also experience an endothelial dysfunction post-procedure similar to catheterized radial arteries, which may trigger vascular complications. Therefore, endothelial function in the catheterized

peripheral artery may represent the coronaries' endothelial dysfunction and consequently the effects of cardiac events, in-stent restenosis and revascularization procedures. Given the above evidence, I believe that further studies are required to examine the recovery of FMD following catheterization procedures and whether FMD post-PCI in the catheterized arteries could predict future vascular complications. Similar study design to that used in **Chapter 4** should be used to examine the FMD response at 2 weeks, 1 month and 2 months after catheterization.

Subsequently, our observation of preserved exercise-induced vasodilation 1 week post catheterization **(Chapter 4)** suggests that exercise is safe following endothelial disruption. Together with two large-scale studies which demonstrated that performing exercise a few days following PCI did not induced cardiac events [302, 303], early exercise-based rehabilitation following catheterization seems appropriate. Given the clear evidence of improved endothelial function following exercise training in CAD patients [26, 37], I suggest that the next step should be to design studies assessing whether early exercise rehabilitation (starting from 1 week post PTCA and/or PCI) can counteract the impact of catheterization and lead to earlier recovery and/or higher endothelial function (FMD) compared to non-exercised patients. So far, there is only one study investigating the effect of exercise post-catheterization. The study demonstrated that 6 weeks of handgrip exercise resulted in improved FMD in the exercise group, whereas FMD remained decreased in non-exercised controls [163].

In addition, given the benefits of exercise on endothelial function are systemic, and not specific to the exercised limbs [25], it is vital to examine the effects of exercise training in the non-exercised limbs following arterial injury. For instance, examining the effects of

cycling on endothelial function in the catheterized radial artery, may provide important information about how coronary arteries recover following PTCA and/or PCI. From a clinical prospective, such information will be essential, as coronary artery endothelial dysfunction has been correlated with a higher degree of intimal thickening in animals [174] and higher lipid core plaques in the vascular walls of patients [403]. Importantly, we have already obtained ethical approval (REC reference: 13/NW/0088) to a) examine the catheterized radial artery response to acute systemic cycling exercise bout (three, 5-min stages at 20%, 40% and 60% of Heart Rate $(HR)_{max}$) and b) the effect of 8-week cycle exercise training (40-70% of target HR_{max} – Kornoven formula adjusted for the effect of HR limiting medication) starting 7-10 days post-catheterization on catheterized and contralateral radial artery FMD. Both testing and supervised exercise training will take place in the LHCH to handle better any exercise-related adverse event. However, due to difficulties related to time, equipment and the necessity to involve more researchers and clinicians for the supervised training sessions, I did not undertake this study within the PhD timeframe. We strongly believe that given the encouraging data of preserved dilation in response to local handgrip exercise following catheterization, the next step is to examine whether cycling exercise, which involve systemic response to greater extent would improve and fasten the recovery of endothelial function post-PCI.

8.3.2. EC expression of atherogenic-modulating proteins in different CAD stages.

To our knowledge, our data is the first to suggest a positive association between endothelium-dependent dilation and eNOS Ser^{1177} phosphorylation **(Chapter 5)**. The data also showed no correlation between endothelium-dependent dilation and markers of oxidative stress. Importantly this data comes from a group of patients with established CAD. In patients without established CVD, such as healthy ageing, previous studies have shown

an inverse association between endothelium-dependent dilation and markers of oxidative stress [88] and no association between endothelium-dependent dilation and eNOS Ser^{1177} phosphorylation [76]. This suggests that the mechanisms which drive endothelial dysfunction in patients with CAD are different to individuals without established CVD, with further elevations in oxidative stress becoming less important to progressive declines in endothelium-dependent vasodilation, and maintaining eNOS Ser^{1177} phosphorylation becoming increasingly important. Future studies should investigate this hypothesis in more detail. I would suggest a study in which a large range of participants, with and without established CAD, are recruited. This would allow us to see how protein content and phosphorylation of these proteins are modified across atherosclerotic development. In addition, endothelial protein expression of oxidative markers and eNOS (content and activation status) should be evaluated following exercise training in both CAD patients and healthy and/or age-matched controls to examine whether the above hypothesis of maintaining eNOS Ser^{1177} phosphorylation and not oxidative stress is the predominant mechanism leading to vascular adaptations in CAD patients.

8.3.3. Does PECAM-1 have a role as a mechanosensor in humans?

Data from cultured ECs suggests that PECAM-1 acts a part of a mechanosensing complex, and that Tyrosine phosphorylation of PECAM-1 is essential for eNOS phosphorylation. However, **Chapter 7** and previous work in humans have shown that PECAM-1 is not phosphorylated following prolonged shear stress in humans, questioning PECAM-1's role as a mechanosensor. Interestingly, Bogi *et al.* [398], observed that only the initial dilation of vessels (up to 120 seconds) in response to shear stress was impaired in muscle arterioles isolated from PECAM-1 knockout mice compared to wild type mice. This observation suggests that PECAM-1 is involved in the initial response to shear stress. Given that these

human studies in the skeletal microcirculation [284] and in conduit arteries **(Chapter 7)** collected ECs following a prolonged period of elevated shear stress (20-30 minutes), it is possible that both studies have missed the period where PECAM-1 may become activated in response to shear. Therefore, at this point I believe further human studies should examine PECAM-1 phosphorylation status at different stages of shear stimulus (initial vs prolonged). This would clarify whether PECAM-1 is activated at the onset of shear stress or if it is not involved in mechanosensing in humans at all.

In addition to the above, another attractive research direction would be to examine eNOS Ser^{1177} phosphorylation in response to different shear patterns or smaller elevations in shear stress. For example, both our study **(Chapter 7)** and Casey *et al.* [66] reported increased eNOS Ser^{1177} phosphorylation in ECs following a ~5-fold increase in shear stress. This generates further questions such as whether lower levels of shear stress, for instance, in forearm arteries during leg cycling (~2-3 fold increase in brachial artery flood flow [404]), could also activate eNOS, and whether such activation is dose-dependent. If this is the case, upregulation of eNOS Ser^{1177} may be the mechanism thought which exercise improves endothelium-dependent dilation in non-exercised limbs [25].

8.4. Final Conclusion

This PhD thesis proposed three specific aims: a) to provide evidence about the impact of catheterization-induced damage on vascular function, b) to examine relationships between specific atherogenic-modulating proteins expressed in endothelial cells, including eNOS and its activation, and endothelium-dependent dilation, and c) to explore the mechanisms relating to acute exercise and NO production. **Chapters 4 and 6** demonstrate that catheterization-induced damage results in impaired endothelium-dependent dilation, and

such responses may be exaggerated in subjects with a fully functional endothelial layer compared to patients with *priori* endothelial dysfunction. In contrast, exercise-induced vasodilation appeared to be preserved following endothelial damage in CAD patients **(Chapter 4)**, highlighting that vascular responses to catheterization may be stimuli specific. Preserved exercise-induced vasodilation suggests that early exercise-based rehabilitation (1 week) following catheterization procedures is safe, although this should be confirmed in other cohorts and with a larger sample. **Chapter 5** provided insights into the molecular mechanisms underlying the progressive development of endothelial dysfunction in CAD patients, with the purpose of helping to understand the vicious cycle of atherosclerosis and endothelial dysfunction apparent in CAD patients. Importantly, we demonstrated that FMD was positively related to eNOS Ser^{1177} phosphorylation and markers of senescence (p21, p16), which together with the lack of correlation between endothelium-dependent dilation and markers of oxidative stress, suggest that different mechanisms may underlie the progression of endothelial dysfunction in CAD patients. Lastly, **Chapter 7** showed that acute exercise increased eNOS Ser^{1177} phosphorylation, but not PECAM-1 Tyr^{713} phosphorylation, in arterial ECs of young healthy well-trained males, suggesting that PECAM-1 does not play a role in shear stress sensing in response to prolonged exercise.

In summary, this “bench to bedside” thesis has combined state-of-the-art molecular biology techniques, with direct *in vivo* harvesting of endothelial cells and compared the results to high resolution imaging during functional arterial bioassay in humans. The results have enhanced current knowledge relating to the impacts of the disease processes (atherosclerosis, endothelial dysfunction) that underlies the world’s most common causes of death (CAD, ischaemic stroke), along with the impacts of the most prevalent strategies to manage this disease (interventional cardiology, catheterization). The outcomes will inform

fields including cardiology, vascular physiology and exercise science and provide important guidance for future studies.

CHAPTER 9 – References

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