Carotid Artery Reactivity: A potential novel, non-invasive test of endothelial function to predict post-cardiac surgery outcomes, cardiovascular events and survival

A thesis submitted to Liverpool John Moores University for the degree of Doctor of Philosophy

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1. Table of Contents

List of Tables and Figures

standardization tools for 1: standardization for diameter measurement and 2: standardization for pulse-wave velocity calculation and C) Demonstrates the carotid artery response to the cold pressor test including 1: Baseline diameter recording and 2: Whilst the hand is submersed in icy-cold water.

Study 1

Table 2.

A table of demographic data of all participants involved in the trial. Demographic data is expressed as mean with SD.

***Figure 7*.**

Mean and Standard Deviation participants during the cold pressor test (CPT) A) Mean arterial pressure (MAP) response to the CPT and B) Diameter response to the CPT. One-Way ANOVA performed to compare baseline vs increase in both MAP and diameter.

***Table 3*.**

Demographic data of participants separated according to their age into young (n=26) and older (n=14) subjects including SD.

***Table 4*.**

Mean Arterial Pressure and Diameter responses in participants at baseline and average MAP throughout the CPT in participants when separated according to "young" (30-45, n=26) and "older" (46-60, n=14)

***Table 5*.**

Statistical comparison of "old" vs "young" baseline, average and max MAP and CAR% throughout the CPT. "Young" defined as aged 30-45 (n= 26) and "old" defined as 46-60 (n=14).

Table 6.

A list of R2 values investigating the Pearson linear relationship between the average increase in MAP throughout the CPT when compared to baseline MAP expressed as a percentage (AVGMAP%) compared to cardiovascular risk factors.

Table 7.

CAR% and blood pressure results of the groups divided based on relative ΔMAP (low= <15%, medium = between 15% and 30%, high = >30%). Relative ΔMAP is the percent increase from baseline to the peak MAP. A) Post-hoc significantly different from group 1 B) Post-hoc significantly different from group 2.

Table 8.

Mean plus SD MAP and Diameter data of participants when split into groups according to their ΔMAP (low= <15%, medium = between 15% and 30%, high = >30%). Relative ΔMAP is the percent increase from baseline to the peak MAP.

***Table* 9.**

Statistical comparison of MAP and Diameter during baseline, AvgCPT and MaxCPT in participants grouped "low", "medium" and "high" according to their ΔMAP.

***Table* 10.**

Participant characteristics and CAR% when divided into groups based on the presence of diameter dilation or vasoconstriction. P-values refer to an unpaired t-test.

***Table* 11.**

Correlation between CAR% and the blood pressure variables during CPT for all participants and the dilation group. ΔBP is the absolute difference between peak and baseline blood pressue, whereas relative ΔBP is the percent increase from baseline to the peak blood pressure.

Study 2

***Figure 8*.**

A CONSORT diagram of recruitment process, enrolment and outcomes.

Table 12

A summary table of studies which have investigated the predictive capacity of endothelial function

***Table* 13.**

Group characteristics of participants when grouped according to the presence or absence of Adverse Events

Table 14.

A table of the carotid artery response in whole-group and in AE and non-AE groups.

***Table* 15.**
Family History and known risk factors in AE and non-AE groups.

***Table* 16.**
A Table investigating the effect of disease severity in adverse event and non-AE groups.

***Figure* 9.**

A comparison of EuroSCORE II in participants in adverse event and non-AE groups.

***Table* 17.**
Characteristics of participants when separated into those with greater than, and less than median CAR% (1.96%)

***Table* 18.**
Effect of Family History and known risk factors in those with a CAR% above, and below median CAR%.

***Table* 19.**

A Table investigating the effect of disease severity in those with a CAR% above, or below median.

Figure 10

A comparison of EuroSCORE II and CAR% in participants when they are dichotomized according to their CAR%.

***Figure 11*.**

Relationship between CAR% and EuroSCORE II. "Positive AE" defined as participants who suffered an adverse-event post-operatively.

***Table* 20.**

A table investigating the number of adverse events in dichotomized participants. A one-tailed analysis was performed with a 95% confidence interval.

***Figure 12*.**

A ROUT analysis investigating the presence of outliers within the dataset. This identified three outliers in which two had adverse events (blue) and one did not (red).

***Figure* 13.**

A logistic regression analysis of the ability of Eurscore2 to predict adverse events in CABG patients.

***Figure* 14.**
A logistic regression analysis of the ability of CAR% to predict adverse events in patients undergoing CABG surgery.

***Figure* 15.**
A multiple logistic regression analysis of the ability of CAR% combined with EuroSCORE II to predict adverse events in CABG patients.

Chapter 7 – Thesis Conclusions and Future Work
Figure 16.

A diagrammatic representation of the vascular response throughout the three phases of the CPT. Including the "baseline", "vasodilation" phase through SNS stimulation and the "recovery" phase. A) A normal dilation phase in which both proximal and distal walls dilate equally throughout the vasodilation phase and B) A non-uniform dilation phase in which the distal wall moves to a greater extent than the proximal wall.

***Figure 17*.**

A diagrammatic representation of the process of vascular ultrasound using B-mode 2D ultrasound. A) A normal straight artery in which all areas of the inner wall of both the proximal and distal aspects of the artery are within the area of maximum resolution and B) A tortuous artery in which the proximal aspect of the inner wall are partially outside the area of maximum resolution producing sub-optimal scan resolution.

Appendix 1

***Table* 21.**

A PICO table describing the search framework used as part of the search strategy.

***Figure 18*.**

A Prisma diagram of the available literature on coronary artery endothelial function and endothelial function in risk prediction.

Appendix 2

***Figure* 19.**

Scatterplots of Age, Sex, CCS score and NYHA score. Participants in grey had post-operative adverse events.

***Figure* 20.**

Scatterplots of CAR% vs BMI. A) A correlation of CAR% vs BMI in this group. There appears to be a number of "clusters". B) CAR% in participants when grouped according to their BMI group according to NHS guidelines for BMI. Grey data points are those who had adverse events. Black data points had no adverse events.

***Figure* 21.**
Scatterplots of Family History and Risk factors for developing cardiovascular disease and adverse events in CABG surgery. Grey data points are those who had adverse events. Black data points had no adverse events.

***Figure* 22.**

Scatterplots of markers of disease severity. Grey data points are those who had adverse events. Black data points had no adverse events.

List of Abbreviations and Acronyms

13N	Nitrogen-13 - radioactive isotope
Ach	Acetylcholine
AE	Adverse Events
ALDH	(mitochondrial) Aldehyde Dehydrogenase
AUC	Area Under Curve
AVGMAP	Average MAP
AVGMAP%	Average MAP expressed as a percentage
BART	Brachial Artery Reactivity Testing
BMI	Body Mass Index
BP	Blood Pressure
CABG	Coronary Artery Bypass Graft
CAD	Coronary Artery Disease
CAR	Carotid Artery Response
CAR_{90}	CAR% at 90 seconds
CAR_{AUC}	CAR% area under curve
CBF	Cerebral Blood Flow
CCA	Common Carotid Artery
CCS	Canadian Cardiovascular Society - A clinical score of angina
cGMP	Cyclic Guanosine Monophosphate
cIMT	Carotid Intima-Medial Thickening
cm	Centimeters
CO^2	Carbon Dioxide
CPT	Cold Pressor Test
CT	Computed Tomography
CV	Cardiovascular

CVD	Cardiovascular Disease
DEF	Dynamic End-Tidal Forcing - a tool used to control breathed CO2
ECG	Electrocardiogram
eNOS	endothelial Nitric Oxide Synthase
EPR	Electronic Patient Records
FAI	Fat Attenuation Index
FDR	False Discover Rate
FMD	Flow Mediated Dilation
GI	Gastrointestinal
GP	General Practitioner
GTN	Glycerol Trinitrate
H^+	Hydrogen ions
$H_{1/2}$	Hypothesis 1/2
HES-ONS	Hospital Episode Statistics and Office of National Statistics
HRA	Health Research Authority
ICU	Intensive Care Unit
IMT	Intima-Media Thickening
iNOS	inducible NOS
Kg	Kilogram
Kg/m^2	Kilograms Per Metre Squared
LAD	Left Anterior Descending Artery
LHCH	Liverpool Heart and Chest Hospital
LJMU	Liverpool John Moores University
MAP	Mean Arterial Pressure
MBF	Myocardial Blood Flow
MHz	Megahertz

MI	Myocardial Infarction
MINS	Myocardial Injury After Non-Cardiac Surgery
MM	Medical Management
mmHg	mm of Mercury - a measure of pressure
MRI	Magnetic Resonance Imaging
NACSA	National Adult Cardiac Surgery Audit
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Centre for Biotechnology Information
NHS	National Health Service
NICE	National Institute for Clinical Excellence
NICOR	National Institute for Cardiovascular Research
NID	Nitroglycerin Induced Dilation
nNOS	neuronal NOS
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NYHA	New York Heart Association - A score of dyspnoea
O:E	Observed vs Expected
$_{PA}CO2$	Partial Pressure Of Arterial CO2
PAD	Peripheral Arterial Disease
PCI	Percutaneous Coronary Intervention
PET	Positron Emission Tomography
$_{pet}CO2$	Partial Pressure Of End Tidal CO2
PICO	Population, Intervention, Comparison, Outcome - Literature search framework
PPI	Public and Patient Involvement
QRISK3	A Clinical Risk Score of developing CVD
RBC	Red Blood Cell

RH-PAT	Reactive Hyperemia Peripheral Arterial Tonometry
RHI	Reaction Hyperemia Index
ROC	Receiver Operated Characteristic
ROI	Region Of Interest
ROS	Reactive Oxygen Species
sGC	soluble Guanyl Cyclase
SMC	Smooth Muscle Cell
SNS	Sympathetic Nervous System
SPET	Single Photon Emission Computed Tomography
SS	Shear Stress
STS	Society for Thoracic Surgeons
SURE	Service Users Research Endeavour
TIA	Transient Ischaemic Attack
UK	United Kingdom
UREC	University Research Ethics Committee
VSMC	Vascular Smooth Muscle Cell
ΔBP	Change in BP
ΔMAP	Change in MAP throughout CPT

Publications

Peace, A. Van Mil, A. Jones, H. Thijssen, D. 2018. Similarities and differences between carotid artery and coronary artery function. Current Cardiology Reviews. 2018. 14(4): 254-263.

Peace, A. Pinna, V. Timmen, F. Speretta, G. Jones, H. Lotto, R. Jones, I. Thijssen, D. 2019. Role of blood pressure in mediating carotid artery dilation in response to sympathetic stimulation in healthy, middle-aged individuals. American Journal of Hypertension. 2019. 33(2):146-153.

Conference Presentations/Posters

Liverpool John Moores University Research Festival, Liverpool. Poster Presentation. (2018, 2019)

Liverpool John Moores University Faculty of Education, Health and Community Faculty Research Day. Oral Presentation. (2018)

Liverpool John Moores University Institute for Health Research Oral Presentation (2018)

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National Symposium on Biomechanics in Vascular Biology and Cardiovascular Disease. QMUL/UCL. Poster Presentation. (2019)

Thesis Abstract

Introduction.

In healthy individuals, coronary arteries dilate in response to sympathetic nervous system (SNS) stimulation, whereas in patients with increased cardiovascular disease (CVD) risk this produces a truncated dilation. Conversely, in patients with significant disease, these arteries constrict. Previous research has demonstrated a correlation between carotid and coronary artery function. In addition, a non-invasive measure of carotid artery response (CAR%) has been shown to predict adverse events in patients with peripheral arterial disease. In this thesis, these findings were extended to investigate the effect of blood pressure (BP) increase caused by the Cold Pressor Test (CPT) as a driver of CAR%. CAR% was then also investigated as a means to predict risk in patients with central arterial disease undergoing Coronary Artery Bypass Graft (CABG) surgery.

Methods. The (CPT), a sympathetic nervous system stimulus (SNS) using cold water, was used to investigate carotid artery response to an SNS stimulus in two studies. The first investigated CPT alongside continuous blood pressure measurement. The second examined CAR% in patients with CVD undergoing CABG, with a 30-day follow-up period.

Results. **Study one:** The timing of peak CAR% was significantly later than peak MAP. There was no significant difference in CAR% when participants are separated by MAP response. There was no significant difference in MAP in “dilation” vs “constriction” groups. **Study two:** There was a weak inverse correlation between CAR% and EuroSCORE II. Neither EuroSCORE II nor CAR% alone predicted risk. There may be a trend towards a marginal improvement in predictive capacity when CAR% is combined with EuroSCORE II. Although this did not predict risk.

Discussion. **Study one:** This study provided evidence that CAR% is not significantly driven by increase in MAP throughout the CPT. In addition, it further supports the validity of CAR% as a surrogate of endothelial function. **Study two:** Alone, neither EuroSCORE II nor CAR% were predictors of risk. Combined, the two measures demonstrated marginal increased predictive capacity. This study suggests that CAR% may improve the predictive capacity of established prediction tools. This cannot be tested conclusively due to the low number of adverse events.

Future Work: This thesis highlighted future areas for research including regulation of CAR%, areas for further technological and practical development and areas of potential clinical integration, namely in the identification and monitoring of endothelial function alongside interventions to improve function and reduce perioperative risk.

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1. Chapter 1: Introduction

The central aim of this thesis is to investigate a novel measure of carotid artery endothelial function as a surrogate of vascular health. This test is investigated in both healthy participants and in patients with coronary artery disease (CAD) undergoing cardiac surgery.

The thesis is presented in seven chapters. The first chapter provides a general introduction to the thesis.

In the second chapter, the background to the importance of endothelial function, endothelial dysfunction and the physiological implication of the endothelium in arterial health are discussed. Current methods of measuring endothelial function, including both invasive and non-invasive measures of endothelial function are described. The direct and indirect measures of coronary artery endothelial function are also discussed. Together, this provides the basis for the thesis whereby carotid artery response (CAR%) may be uniquely placed to offer an insight into coronary artery endothelial function.

The third chapter details the methodology employed within this thesis, focusing on the use of the cold pressor test (CPT) as a method to stimulate the sympathetic nervous system (SNS) response, alongside the use of carotid artery ultrasound as a tool to investigate carotid artery diameter.

The fourth chapter outlines the statistical analyses performed, including the justification for the analysis, the decision-making process and any limitations and considerations with these statistical measures.

The fifth chapter describes study one, where the relationship between blood pressure (and therefore transmural pressure) and carotid artery response in healthy middle-aged individuals was investigated. The extent to which transmural pressure affects carotid artery diameter in response to sympathetic stimulation is currently unknown. This is key as the CPT was employed to investigate endothelial function as a surrogate of vascular health.

The sixth chapter described study two. In this, CAR% was investigated in pre-operative patients undergoing Coronary Artery Bypass Graft Surgery (CABG) combined with Valve Surgery. This was used to investigate the relationship between CAR% and current established methods to predict risk. The relationship between CAR% and adverse events (AE) was also investigated to investigate if CAR% was predictive of AEs in this group. The CAR% may offer a non-invasive method to investigate carotid artery response as a surrogate of coronary artery function in surgical patients. Coronary artery endothelial function has previously been demonstrated to predict disease progression and outcomes in patients with established cardiovascular disease (CVD).

The seventh and final chapter provides a discussion of the future work required to fully understand the regulation of CAR%. It also highlighted potential areas for further development in order to improve the accuracy and reliability of CAR% as a measure of endothelial function. It also discusses the potential clinical implications of CAR% and how it could be incorporated into clinical practice, namely CAR% as a method to easily identify endothelial dysfunction. This may be applied to improve perioperative risk prediction. The CAR% may also be used to quantify endothelial dysfunction and therefore offer a target for treatment which aims to improve endothelial function in clinical groups.

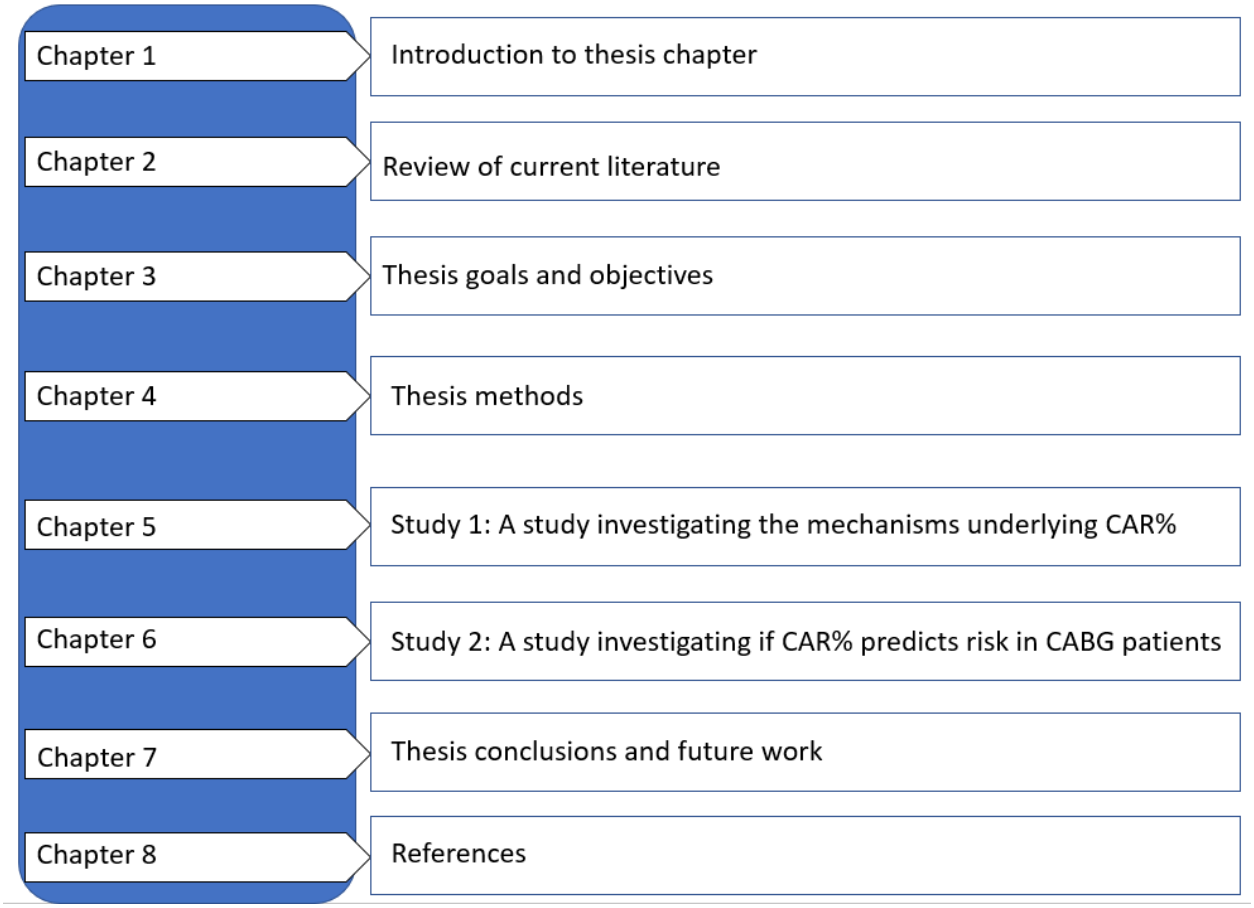


Diagram 1) A schematic representation of chapters contained in this thesis.

2. Chapter 2: Background

2.1. Chapter introduction

This first chapter introduces the process of atherosclerosis, coronary artery disease as well as CABG surgery as a treatment for coronary artery disease. This also outlines current methods of risk prediction. This then further discusses endothelial function, current techniques in investigating endothelial function and the current literature investigating endothelial function as a method to predict risk in coronary artery disease patients. Finally, this chapter discusses the limitations in current methods used to investigate endothelial function and explores the use of CAR as a method to quantify endothelial function and predict risk in patients undergoing CABG surgery.

2.2. Coronary Artery Disease

Cardiovascular disease (CVD) is an umbrella term that describes disease of the heart and blood vessels, and remains the world's leading cause of morbidity and mortality (1). CVD accounts for approximately 17.7 million deaths world-wide each year, or approximately 31% of all deaths (2). Coronary Artery Disease (CAD) is a sub-type of CVD. Coronary artery disease is disease of the blood vessels which supply blood to the heart muscle (myocardium). Despite improvements in clinical management, and an associated reduction in CVD-related mortality (3), the incidence of CVD continues to rise (3). An ageing population, and an increase in prevalence of risk factors in younger adults (e.g. obesity, diabetes and hypertension) are likely to contribute to this trend (4). Coronary Artery Disease has a profound impact on both individuals and their families. CAD has been associated with a significant decrease in quality of life with a progressive decrease in quality

of life. This includes both disability and symptom-associated quality of life (5, 6) and a resultant significant decrease in mental wellbeing and increase in mental health issues (5, 6) . As well as a significant burden on the individual, CAD has a significant societal impact. The 5-year cost of a cardiovascular disease in high-risk patients is £23,393 and lifetime cost of £116,888. The total cost of CVD-related healthcare costs in England is estimated to be £7.4 billion per year with an estimated cost to the wider economy of £15.8 billion per year (7). This therefore means that coronary artery disease and cardiovascular disease more broadly have a huge impact for the individual, their families and society as a whole.

Coronary Artery Bypass Graft Surgery

A common treatment option for CAD is Coronary Artery Bypass Graft (CABG) surgery, with over 800,000 operations undertaken world-wide each year (8). Approximately half of which are performed in North America and one quarter performed in Northern Europe (8). CABG surgery is an important treatment option offered to patients with significant coronary artery disease on the grounds of both symptom relief and prognostic benefit (9) .

In CABG surgery, flow-limiting lesions are first identified using angiography or cardiac MRI. The operation is typically performed through a mid-sternal incision. During the operation, potential targets are sought and identified distal to the flow-limiting lesion (distal anastomosis). A graft (conduit) is then taken from elsewhere within the body. These are typically the internal thoracic artery or gastro-epipolic artery although this is less commonly used. The use of these arteries do not require proximal anastomosis. The saphenous vein may also be harvested and used, although this requires both distal and proximal anastomosis. In both cases, these are used to bypass the

flow-limiting lesions thereby restoring blood flow to ischemic segments of myocardium. This can be used to restore blood flow in all of the major coronary arteries, depending upon clinical need. This is typically performed in the arrested heart, whilst blood oxygenation and circulation is managed by cardiopulmonary bypass. A smaller number of specialist "off-pump" CABG procedures are also undertaken in the absence of circulatory arrest.

Although CABG surgery is a common treatment for CAD, it is associated with significant risk. Cumulative short-term risk of operative mortality in isolated CABG surgery is approximately 2.5% (10). The longer-term survival in these patients reduced significantly with 5 year survival-, 15 year survival-, 25 year survival -, and 35 year survival -, being 86% ± 0.3%, 48% ± 0.5%, 19% ± 0.6%, and 7% ± 1.2% respectively (10). The National Adult Cardiac Surgery report investigates morbidity and mortality data within the UK over the last 3 years between 1st April 2015 and 31st of March 2018 (National Adult Cardiac Surgery Audit (NACSA) dataset (version 5.1.0)). This is a UK-specific dataset. In 2017/18 there were 32,295 operations undertaken. The mortality rate of isolated CABG surgery is approximately 1-2%. The 5, 10 and 15 year mortality in isolated CABG surgery between 1999 and 2017 in the UK is 9.2%, 23.5% and 35.6% respectively(11). CABG surgery is performed mostly in isolation. However, CABG surgery is also performed in combination with other cardiac and non-cardiac surgeries. CABG surgery alongside valve surgery is the most common type of combined CABG surgery. The combination of CABG combined with valve surgery is associated with a further increase risk of mortality (12). This makes CABG surgery a commonly undertaken surgical intervention with significant operative and post-operative risk. Although clinical knowledge and operative ability continue to improve, the increase in age and comorbidities mean that an increased number of higher-risk operations are being undertaken.

This increase in the number of higher risk increasingly complex surgeries highlights the requirement to accurately risk stratify patients perioperatively (4).

R**isk Prediction in Coronary Artery Disease and CABG Surgery**

Traditionally, predicting future development of CAD and cardiovascular events in CAD patients was based on the evaluation of (non)modifiable risk factors, such as age, gender and family history of premature CAD. Alongside modifiable risk factors including blood pressure, cholesterol, body weight, and glucose homeostasis (13). These risk factors are associated with development of cardiovascular disease, disease progression in patients with established CAD and AEs in patients undergoing surgical intervention (14). Although these risk factors have been associated with increased risk in large scale studies, the precise mechanisms are poorly understood. The impact of traditional risk factors on atherosclerosis may at least partly be explained through their direct effects on the endothelium. For example, elevated cholesterol, high blood pressure, smoking, ageing and obesity are all associated with an increased risk of atherosclerosis (15, 16). This is typified by endothelial dysfunction. Endothelial dysfunction also precedes atherosclerosis.

Molecular mechanisms of CAD

It is thought that at least in part, CVD risk factors negatively affect endothelial function through an increase in ROS. The molecular mechanisms and consequences aren't completely understood. However, some of the mechanisms and consequences have been investigated. The consequences can be broadly categorised in to dysfunction affecting eNOS, a reduction in NO bioavailability and abnormal responsiveness of VSMC's to NO.

Dysfunction of eNOS.

A significant cause of endothelial dysfunction is associated with a dysfunction in eNOS activity. These are briefly outlined below. This is reviewed more extensively in previously published articles (17, 18) .

Reduced Substrate Activity

L-arginine is the key precursor to NO. In healthy adults, this can be synthesized de-novo from L-citruline. When there is a reduction in the availability of both L-arginine and L-citruline, this can contribute to a reduction in bioavailability of NO. The acute supplementation of L-arginine can curtail endothelial dysfunction, although this benefit may not be seen chronically (17, 19). The increase of endothelial arginases associated with aging, diabetes or hypertension competes with eNOS for L-arginine thereby reducing bioavailability of NO (20-22) .

Reduced cofactor availability

A key cofactor in NO production is BH_4 from sepiaterin. If there are low circulating levels of BH_4 or if there is an increase in oxidation of the precursor, BH_2 (23). This is associated with a reduction in NO bioavailability. The reduction in BH_4 or increased oxidation of BH_2 is associated with diabetes mellitus (24), prolonged exposure to aldosterone or cortisol associated with hypertension (25), in chronic inflammation (26) or in post-menopausal women (27) . Importantly, supplementation with exogenous BH_4 improves endothelial function in patients with BH_4-deficient (23, 28).

Increased Endogenous Inhibitors

Asypometric dimethylarginine (ADMA) preferentially displaces an L-arginine precursor thereby inhibiting NO production. The increase in ADMA can be accelerated by increased oxidized stress

augmented by levels of oxidized and carbamylated LDL. The increase in endogenous inhibitors thereby reduce NO production(17).

Reduced Protein Presence

The reduction in gene expression encoding eNOS results in a reduction in NO production. CVD risk factors including aging, diabetes mellitus, or hypertension result in reduction in the presence of proteins associated with NO production (17). These risk factors cause decreased protein presence first through the activation of tumour necrosis factor-α causing and translation of elongation factor 1-α thereby destabilizing eNOS mRNA. Second through increased presence of micro-RNA-155 which destabilizes eNOS mRNA. Third by diminished endogenous antioxidant enzymes (catalases) which inhibit activator protein-1 thereby reducing eNOS transcription. Fourth increased levels of oxidized-LDL recruiting myocardin-related transcription factor-A to the nucleus thereby repressing eNOS gene expression (17, 18).

Reduced Protein Dimerization

The dimetric structure of eNOS is essential for its function. There is an increase in monomerization of eNOS observed with aging, diabetes mellitus, hypoxia, hyperlipidemia and increased oxidative stress. These risk factors are associated with S-nitrosylation at Cys94- and Cys99- thereby disrupting the dimetric structure of eNOS thereby reducing NO production (17, 29).

Reduced NO Bioavailability

As well as a reduction in NO production, there is also a reduction in NO bioavailability. The increased presence of superoxide anions with an exaggerated formation of peroxynitrite. This is

the major factor accelerating the disposition of endothelium-derived NO. As well as reducing NO bioavailability, peroxynitrite also has deleterious effects on cell function (18, 30). The blunting of NO-mediated response is observed in obesity, diabetes mellitus and hypertension.

Abnormal Responsiveness of Vascular Smooth Muscle to NO

As well as abnormalities in the production or bioavailability of NO, there is also an alteration in the responsiveness of SMC's to NO. This can be broadly categorized in to reduced vasodilatory response or a paradoxical vasoconstrictive response (18).

Reduced Vasodilatory Response

As well as reductions in endothelial-mediated dilation, resulting in dysfunction in vasodilation, there can also be dysfunction in the context of a normally functioning endothelium. This reduced responsiveness to vasodilatory stimuli can be as a result of a number of distinct mechanisms such as. 1) A reduction in the β-subunit of sGC associated with an increased oxidative stress caused by aging, hypertension, or diabetes mellitus. 2) A decreased responsiveness to endothelium-derived NO caused by an increased expression of phosphodiesterases. These accelerate the breakdown of cGMP, reducing expression of PKG (eg in aging and hypertension) or reducing expression of calcium-gated potassium channels. The net effect is a reduction in hyperpolarization of VSMC's as seen in aging, diabetes mellitus, hypertension and obesity.

In summary, a common mechanism by which traditional risk factors increase cardiovascular risk and increased operative risk in CABG surgery may be through their effect on the endothelium (31). One potential pathway is related to an overproduction of Reactive Oxygen Species (ROS) or increased oxidative stress (32, 33). This contributes to a reduction in NO bioavailability and

promotes cell damage. It has also been recognized that (low-grade) inflammation plays a key role in the development and progression of atherosclerosis and subsequent CAD (34). This is summarized in ***Figure* 1**. Although traditional risk factor analysis is key in risk stratification, the degree to which individual risk factors affect endothelial function is not fully understood. Similarly, the protective effects of exercise and dietary supplementation are not incorporated in to current risk stratification tools (14). This highlights the importance of directly measuring vasomotion as a surrogate of endothelial function, and therefore vascular health in these patient groups. This would allow a move away from epidemiological-derived risk stratification tools to a personalized approach to risk stratification.

The most commonly utilized tool in the UK is the EuroSCORE II tool (35). This was first developed in 1999 with a subsequent major update in 2011. This tool collects 17 items around the clinical status of the patient, state of the heart and operation undertaken and then uses logistic regression analysis to calculate the predicted mortality rate (35) . The EuroSCORE II tool is also used to risk-adjust national cardiac surgery outcome data within the UK (35).

Endothelial Function and Risk Prediction

Coronary artery health has also been suggested as a means to predict future CAD (14). A common method of measuring systemic coronary vascular health is through measuring endothelial structure and/or function (14). The endothelium plays a central role in vascular health and is demonstrated to represent a key step in the initiation and development of atherosclerosis. Located between the circulating blood and the vessel wall, the endothelium fulfills a key role in signal transduction in response to various hemodynamic stimuli, such as shear stress and wall

strain (36) and also contributes to vascular health through the paracrine production of vasoactive substances (37). The key role of the endothelium in the pathophysiological mechanism of CAD is reinforced by the observation that endothelial dysfunction precedes the development of atherosclerosis (38-41), and that measures of coronary artery endothelial function independently predict future CAD (42).

Several techniques are employed to examine endothelial function and vascular structure including Flow Mediated Dilatation (FMD) and carotid artery Inter-Medial Thickening (cIMT) measurement. These methodologies are summarized in ***Table* 1** below. Due to ease of access and their correlation with coronary artery vascular function, these techniques have become common in scientific research (43). The most extensively investigated is FMD. Importantly, brachial FMD has previously been demonstrated to correlate with CAD severity (44), and has been demonstrated to be a prognostic predictor in CAD (45, 46). One potential limitation of FMD is that peripheral arteries show key differences in regulation of vascular tone and anatomy in comparison to coronary arteries (47). This may be why FMD is not yet commonly utilized in clinical practice (48, 49). Techniques utilized to investigate endothelial function are discussed further below. When compared to peripheral arteries, the carotid artery is an easily accessible central artery that is similar in anatomical structure and vasomotor control to coronary arteries (50). The carotid artery may represent a unique opportunity to asses an easily accessible artery with structural and functional characteristics similar to that of central arteries including the coronary arteries.

Table 1) A summary table of both invasive and non-invasive measures of endothelial function. Adapted from Schindler et al (2005), Hays et al (2010), Flammer et al (2012) and Van Mil et al (2018) **(51-54)**

Technique	Vascular Bed	Advantages	Disadvantages	Stimulus (Examples)
Coronary angiography	Coronary arteries	Direct assessment of coronary vasculature Functional and structural analysis Gold standard	Invasive Time consuming Expensive Difficult to perform (requires a specialist)	Acetylcholine Cold Pressor Test
Intima-medial thickening	Peripheral and conduit arteries – often carotid or aorta	Non-invasive Surrogate of coronary structure Easy to perform Fast test	Structural assessment Dependent upon plaque/ increased IMT Added clinical value unknown	
Pulse-wave velocity	Aorta and peripheral arteries (eg radial)	Non-invasive Surrogate for coronary function (aorta) Functional assessment	Limited evidence in periphery Reliability uncertain	Propagation of systolic wave
Flow-mediated dilatation	Peripheral conduit arteries, often brachial/femoral	Non-invasive Surrogacy of peripheral arteries to coronaries	Technically challenging Large between-study variability Inconsistency in FMD value	NO release shear

Carotid Artery Reactivity	Carotid artery	Non-invasive Surrogate for coronary arteries Easy to perform Quick test Demonstrated predictive capacity in peripheral arterial disease patients	Physiology unknown Literature reliant upon coronaries	Cold Pressor Test
Cardiac MRI	Coronary vasculature	Measure of structure and function High resolution images	Technically challenging Prohibitively expensive	Hand grip exercise Nitrates
Positron Emission Tomography	Myocardial Perfusion	Identify risk in angiographically normal patients Measures metabolic demand	Injection of radiolabeled tracers No measure of wall structure	Cold Pressor Test
Single Photon Emission Computed Tomography	Myocardial Perfusion	Identify heightened risk in low-risk patients	Invasive	Exercise/adenosine stress test

2.3. Endothelial Function and Atherosclerosis

The vascular system is lined by the endothelium; a single layer of cells on the inner surface of all vessels. Although the endothelium was first thought of as a simple layer of cells, landmark studies have demonstrated that the endothelium fulfils various important actions (55, 56). Primarily, it provides a semi-permeable barrier between the constituents of the circulating blood and the inner surface of the vessel. This allows the transport of blood-borne cells and macro-molecules across the endothelium, controlled through transport vesicles (e.g. caveolae, vesiculo-vacuolar organelles) and cell-cell junctions (18). The endothelium also has a key function in its ability to regulate vascular tone through the autocrine and paracrine production of vasoactive substances (18). The most important vasodilator produced by the endothelium is nitric oxide (NO). This is a gas with a short half-life of 6-30 seconds within the vessel wall and is produced through the oxidation of L-arginine to L-citrulline. This reaction is catalysed by NO-synthase with cofactors, including tetrahydrobiopterin and nicotinamide adenine dinucleotide phosphate (NADPH). The enzyme endothelial Nitric Oxide Synthase (eNOS) is the predominant enzyme responsible for NO production within the endothelium. Subsequently, NO diffuses to vascular smooth muscle cells (vSMC) and activates guanylate cyclase. This causes an increase in intracellular cyclic guanosine monophosphate (cGMP), leading to relaxation of smooth muscle cells (SMC's) and vasodilation (57, 58). Importantly, NO also exerts inhibitory effects on platelet aggregation, leukocyte adhesion and VSMC migration and proliferation. Through these pleiotropic effects, production of NO contributes to protection against atherosclerosis and cardiovascular disease (18). The complex molecular process of NO production in endothelial function and in dysfunctional

endothelial cells are reviewed extensively elsewhere (18). This is summarized in ***figure* 1**. Taken from Vanhoutte et al (2016) (18).

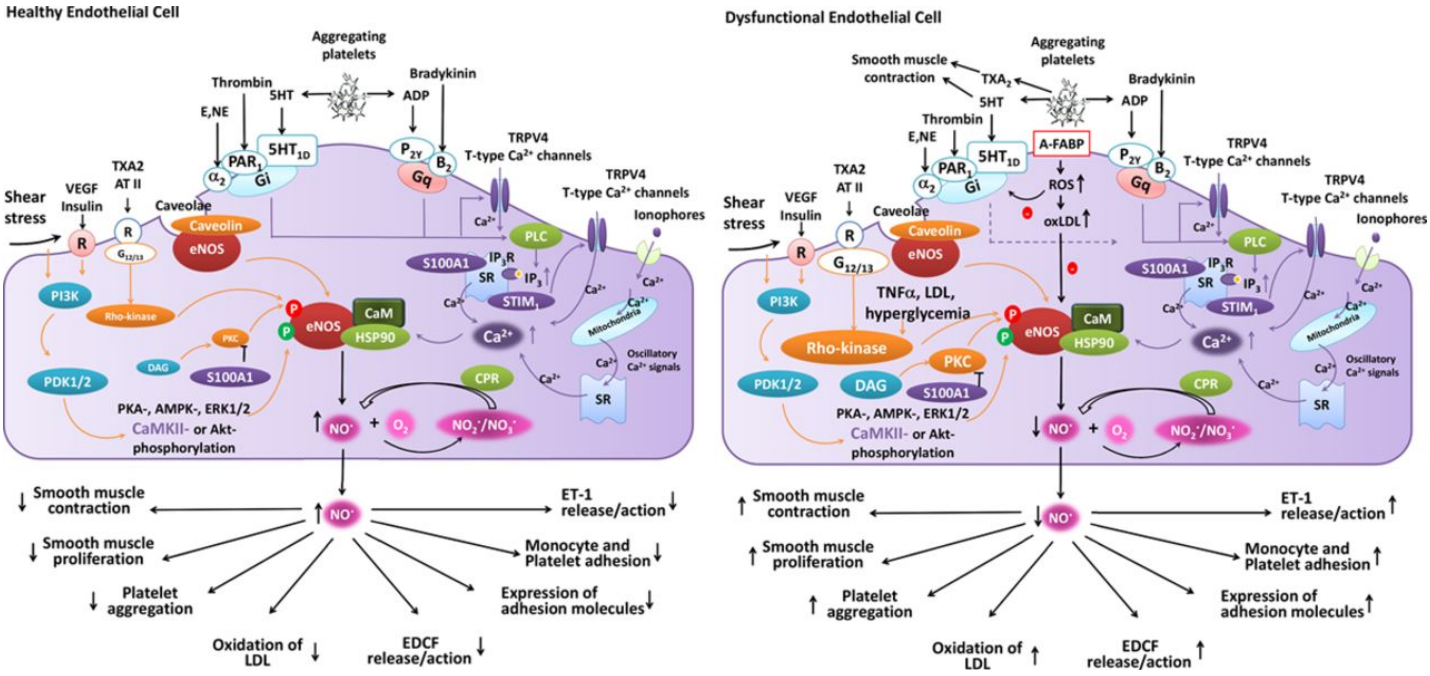


Figure 1) Role of the endothelium in nitric oxide production in both a healthy endothelial cell and in endothelial dysfunction. Taken from (18) with permission.

The process of atherosclerosis is complex and is well documented elsewhere. (16, 59) The relationship between endothelial dysfunction and atherosclerosis is summarized in ***Figure 2*** (60). The impact of traditional risk factors on atherosclerosis may at least partly, be explained through their direct effects on the endothelium. For example, elevated cholesterol, high blood pressure, smoking, ageing and obesity are all associated with an increased risk of atherosclerosis (15, 16). A common mechanism by which traditional risk factors increase cardiovascular risk may be through their effect on the endothelium (31). One potential pathway is related to an overproduction of Reactive Oxygen Species or increased oxidative stress (32, 33). This

contributes to a reduction in NO bioavailability and promotes cell damage. It has also been recognized that (low-grade) inflammation plays a key role in the development and progression of atherosclerosis and subsequent CAD (34).

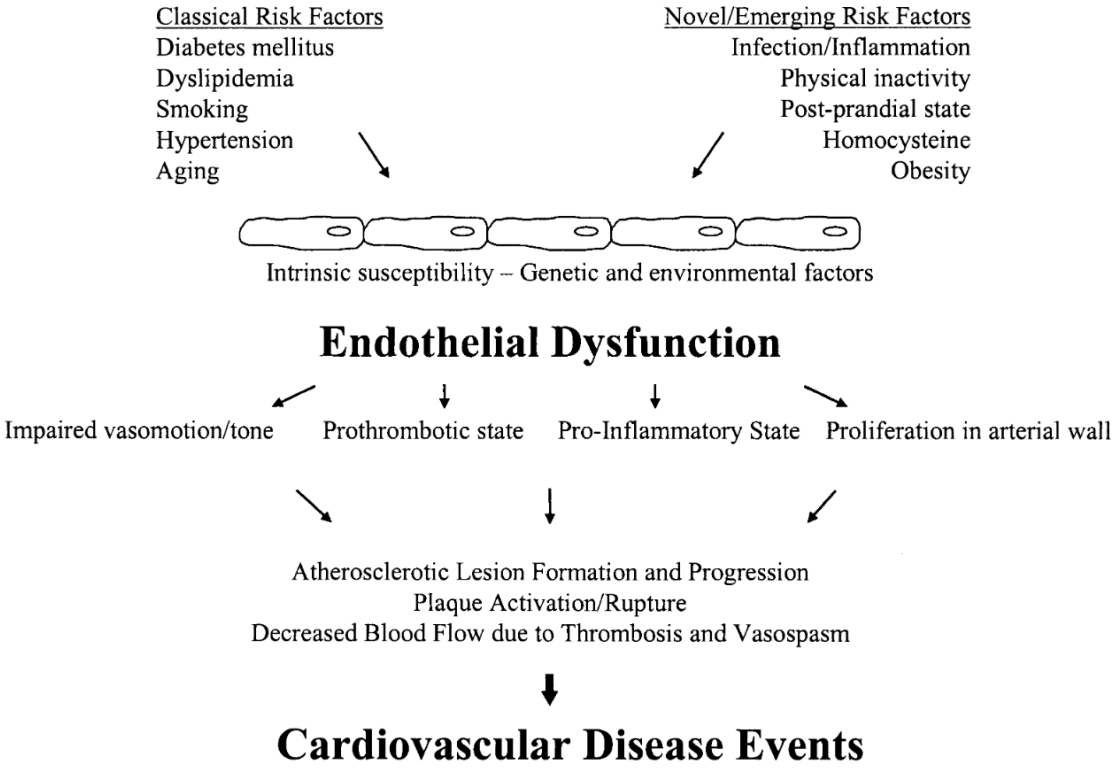


Figure 2) A summary of known and emerging risk factors and the effect on endothelial function and dysfunction and the development of atherosclerotic cardiovascular disease. Taken from (60) with permission.

Previous studies have demonstrated that endothelial dysfunction is an independent predictor of, and contributor to atherosclerosis (61). Ludmer et al (1986) demonstrated for the first time, impaired endothelial function in the presence of atherosclerosis using paradoxical constriction to acetylcholine (Ach) infusion in patients with mild-to-moderate CAD (62). Similarly, endothelial

dysfunction has been demonstrated in pre-clinical stages of atherosclerosis development using procedures involving forearm ACh infusion (63) and brachial artery flow mediated dilatation (FMD) (64). Comparable findings have been presented in subsequent studies (51). This highlights the importance of the endothelium in the development of atherosclerosis.

Coronary Artery Endothelial Function Predicts Future CAD

The strong evidence around the role of the endothelium in mediating atherosclerosis has driven a large field in science, focused on examining whether a measure of coronary artery endothelial function can predict future development of CAD. Schachinger (2000) and colleagues were one of the first to examine whether coronary artery response to endothelium-dependent dilators such as Ach, sympathetic activation, increased blood flow, and endothelium-independent nitroglycerin can predict future development of CAD. They found that impaired endothelium-dependent and -independent coronary responses were independently related to higher incidence of CAD events (42). They followed patients up for 7.7 years. They defined adverse events as death, cardiovascular death (MI, cerebral infarction, sudden cardiac death), unstable angina, CABG, Percutaneous Transluminal Coronary Angioplasty or revascularization of peripheral arteries. Similarly, Suwaidi *et al.* (2000) found that CAD and marked coronary artery endothelial dysfunction was associated with an increased risk of developing CAD (65). In this latter study, endothelial function was measured using intracoronary infusion of acetylcholine along-side coronary artery angiography in 157 patients with mild CAD. They were followed up for a period of 28 months. Similarly, Halcox *et al.* (2002) found that endothelial dysfunction is predictive of cardiovascular events in patients with and without established CAD. This was

investigated using intracoronary infusion of ACh together with quantitative angiography. They included 132 patients with established CAD and 176 participants without cardiovascular disease and a 46 +/- 3 month follow-up (66). They defined adverse events as cardiovascular death, MI, unstable angina, and acute ischaemic stroke. Cardiovascular death defined as, death due to MI or cerebral infarction. Taken together, this suggests that coronary artery endothelial dysfunction is predictive of CAD, independent of the presence of cardiovascular risk factors.

The prognostic effect of endothelial dysfunction was further demonstrated by Suwaidi et al (2000) (52) who found that impaired blood flow in response to SNS stimulation, caused by the CPT and measured non-invasively using Positron Emission Tomography, was predictive of cardiovascular events in 72 patients undergoing diagnostic cardiac catheterization (52). This finding was reinforced by Hays et al (2010) who investigated coronary artery endothelial dysfunction using coronary artery magnetic resonance imaging (MRI). Coronary artery MRI (cMIRI) was used to assess arterial response during handgrip exercise as a sympathetic stimulus. They found marked endothelial dysfunction (i.e. vasoconstriction during handgrip exercise) in patients with established CAD. However, the predictive capacity of this has not been investigated (53). This demonstrates the key prognostic value of coronary artery endothelial dysfunction in CAD risk, and that this can be investigated using both invasive and non-invasive methods. This also highlights the ability to examine vascular responses to endothelium (in)dependent vasodilators as well as activation of the SNS

Endothelial Function and Hypertension

Chronic raised blood pressure (or hypertension) is a key risk factor for CVD. Hypertension is defined as a chronically increased systolic blood pressure above 140mmHg or diastolic blood pressure above 90mmHg at sea level (67). In 2015, it was reported that 1 in 4 adults in England had hypertension (67). This was estimated to contribute to 75,000 deaths (67). There is also an excess all-cause mortality in uncontrolled hypertensive patients (68). Hypertension, cigarette smoking, diabetes mellitus and hypercholesterolemia are all modifiable risk factors associated with an increased risk of CAD (69). Importantly, hypertension has the strongest evidence for causation of CAD within these modifiable risk factors (69). There is also suggestion that biologically normal BP is lower than what has been used in current clinical practice. This may mean that current models underestimate the impact hypertension has on CAD risk. As well as being a significant predictor of risk of CAD, hypertension has also been demonstrated to predict risk of left ventricular hypertrophy, valvular disease, cardiac arrythmias, cerebral stroke and renal failure (69, 70). It is thought that the prevalence of hypertension is approximately 30-45% of the general population (71). Although hypertension is closely linked to CAD and CVD more broadly, hypertension and cardiovascular risk should be interpreted in the context of total cardiovascular risk, rather than the presence or absence of hypertension alone. Although the mechanism isn't fully understood, it is thought that hypertension increased cardiovascular disease risk through an increase in oxidative stress burden. This is reviewed more extensively on in the "molecular mechanisms of coronary artery disease" subheading of this thesis. Hypertension is often considered a "silent killer" as individuals with clinically significant hypertension may remain asymptomatic (72). Chronic hypertension may be at least in part, caused by a loss of peripheral vascular function as well as other mechanisms including alterations in arterial structure and

stenosis. Endothelial function has therefore been extensively investigated in the context of chronic hypertension. Tsutsui and colleagues (2015) demonstrated a significant increase in blood pressure in a neuronal, inducible and endothelial Nitric Oxide Synthase (n/i/eNOS$^{-/-}$) triple knockout mouse model in which all three genes encoding NOS production are non-functioning (73). Similarly, Huang et al (2012) demonstrated a 30 mmHg increase in systolic blood pressure (BP) in a single eNOS$^{-/-}$ knockout model (74). Significant endothelial dysfunction has also been demonstrated in patients with hypertension (75). Dose-response curves to the endothelium-dependent vasodilator Ach were lower in hypertensive patients than in healthy controls (66). However, dose-dependent curves to sodium nitroprusside, an endothelium-independent vasodilator working on the vSMC's, were similar in the two groups. This suggests that the loss of ability of the arteries to dilate in hypertensive patients is due to a loss of endothelial function rather than VSMC-mediated dilatation (66). Importantly, Perticone et al (2001) demonstrated that cardiovascular event rates are higher in hypertensive patients with high-grade endothelial dysfunction compared to hypertensive patients with low-grade endothelial dysfunction (76). This highlights the importance of endothelial function in both CAD and in the risk of developing disease in hypertensive patient

Use of peripheral and central arteries in risk prediction

The majority of research in risk prediction using non-invasive imaging has used the peripheral arteries, particularly the femoral or radial arteries. These are used as surrogates of coronary artery endothelial function. This has been reviewed extensively elsewhere (14). However, there are distinct differences in both the structural and functional characteristics in central vs peripheral arteries. These are summarized in ***Figure* 3**.

The "elastic" arteries have a high density of elastic fibers, lower SMC's, a thin tunica adventicia and a large tunica media with a large number of elastic and collagen fibrils. The net effect of this is the ability of "elastic" arteries to store elastic energy released in diastole to maintain a constant blood pressure above 60mmHg (14) . Whereas the "elastic" peripheral arteries have a large tunica adventicia, a smaller tunica media with less elastic and collagen fibrils. The elastic peripheral arteries also have a large VSMC layer. The net result is an ability to expand and contract as required (14).

A second consideration is in the regulation of vasomotion within both central and peripheral arteries. Although it is incompletely understood, it is thought that CAR% is predominantly driven by adrenoreceptor and metabolic mechanisms. It is thought that shear stress does not play a significant role in carotid artery vasomotion. Whereas, FMD is significantly driven by reactive hyperemia affecting endothelial function (49).

A third consideration in using central vs peripheral arteries as surrogates of coronary artery endothelial function is the age-related changes in different artery types (77). It has previously been demonstrated that, in central arteries there is an increase in arterial stiffness associated

with age and that this is predictive of CVD risk (77). Whereas, age-related peripheral arterial stiffening is attenuated. The value of peripheral arterial stiffening in CVD risk prediction is limited. Arterial stiffness is an important component of vasomotion independent of a fully functioning endothelium (77).

Taken together, the similarities between the carotid and coronary arteries along with the ease of access to the coronary artery through carotid artery ultrasound may mean that the carotid arteries are uniquely placed to offer an easy to access central artery with both structural and functional similarities to the coronary arteries.

2.4. Vascular structure in coronary and carotid arteries

The carotid and coronary arteries are large vessels, often referred to as “elastic” or “conducting” arteries. Both arteries transport large volumes of blood away from the left ventricle to perfuse vital organs, including the brain (partly supplied by the carotid arteries) and myocardium (fully supplied by the coronary arteries). As a result, the walls of both arteries are extremely resilient and contain large volumes of elastic fibres. The tunica media of the coronary and carotid arteries contain a higher density of elastic fibers and fewer SMCs compared to peripheral, muscular arteries (78, 79). These structural characteristics allow elastic arteries to tolerate significant pressure changes that occur across the cardiac cycle. The differences in central “elastic” and peripheral “muscle” arteries are summarized in ***Figure 3***. During ventricular systole, pressure within the vessel rises rapidly and the elastic arteries expand, followed by a reduction in blood pressure during ventricular diastole. The elastic fibers recoil to allow the artery to return to their original dimensions. This expansion during systole dampens the sudden rise in pressure within

the vessels, whilst the recoil during diastole slows the drop in pressure. During this process, elastin in the arterial wall stores elastic energy during systole, and this is released during diastole. Consequently, although blood is pumped from the heart in a cyclic manner, with large differences in systolic and diastolic pressure in the left ventricle, arterial blood pressure in central arteries does not fall below 60-80mmHg (50, 80). In addition to the large content of elastic fibrils, the tunica media also contain collagen fibrils that form a slack network and provide a physical guard against over-distension. As vessels age, the stiffer collagen fibrils increasingly dominate the tunica media within arteries, thereby reducing rebound capacity of elastic arteries. This age-related process contributes to impaired ability to dampen the blood pressure fluctuations (contributing to an increased pulse pressure), but may also contribute to the progression of atherosclerosis and future development of CAD (81, 82).

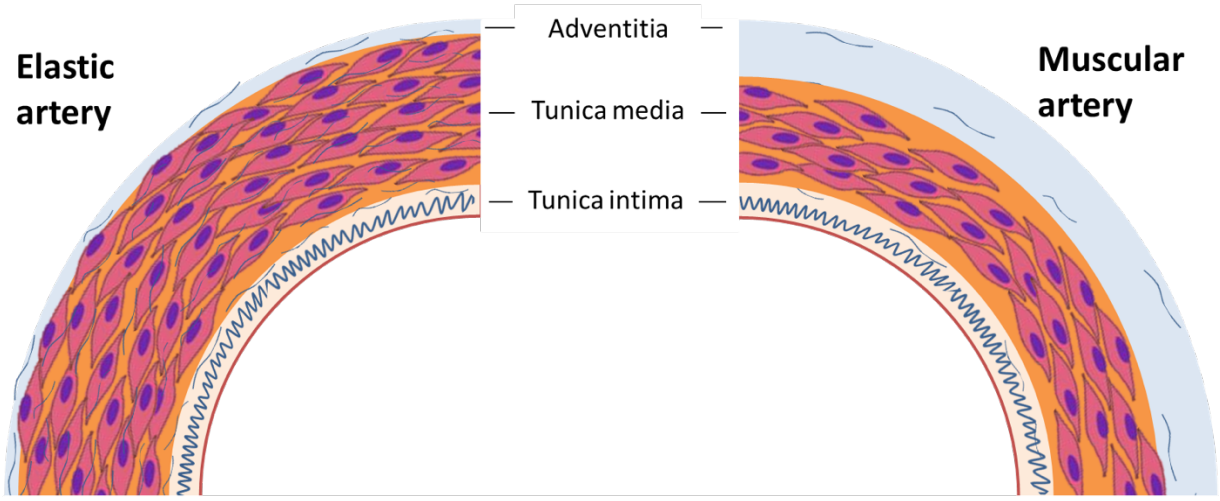


***Figure 3*)** Comparison of anatomical characteristics between elastic (e.g. coronary and carotid) and muscular (e.g. peripheral) arteries. Elastic arteries have a thin tunica adventitia and a large tunica media, containing a large amount of elastic and collagen fibers. Under physiological conditions, there are relatively few vascular smooth muscle cells within the tunica media of the elastic arteries. Muscular arteries have a larger tunica adventitia with a smaller tunica media with

few elastic and collagen fibers, but a large vascular SMC layer. Taken from (14) written as part of this PhD.

Regulation of vascular tone in coronary and carotid arteries

The majority of work examining central artery vascular function is based on knowledge derived from research on the coronary arteries. This is largely because manipulation and infusion of vasoactive substances – both of which are typical procedures to explore function characteristics of arteries, have several limitations when applied to the carotid artery *in vivo*. For this reason, the following section mainly focuses on studies that have examined coronary artery vasomotor function. It is thought that the regulation of coronary artery vascular tone is a result of vasoactive signals influenced by metabolic, neuronal and local mechanisms. All aimed at matching coronary blood supply to oxygen demand.

Metabolic control of vascular tone

A key mechanism of controlling vascular tone in coronary arteries is through the metabolic influence on vascular diameter. Although the mechanism is not fully understood, it is hypothesized that cardiac myocytes produce vasodilatory substances including adenosine, NO, prostacyclin, bradykinin and Carbon Dioxide (CO_2) in response to myocardial oxygen consumption (83). Cardiomyocytes can also produce vasoconstrictive substances including angiotensin II (83). These vasodilatory and vasoconstrictive substances are released in direct response to the oxygen demand and metabolism of local tissues. This allows for a localized response to an increase or decrease in oxygen demand, with the release of vasoactive substances which are directly tied to oxygen requirement and products of metabolism including CO_2, H^+ and lactate (14). Hypoxia can

cause dilatation both directly and indirectly. Hypoxia can cause dilatation indirectly through the release of vasodilatory metabolites. Direct vasodilation through hypoxia may be mediated though the release of tissue metabolites. The formation of CO_2 during increased oxygen demand readily diffuses from parenchymal cells to vSMC's where it causes dilatation. Similarly, hydrogen ions also increase during hypoxia, thereby causing a decrease in pH and vasodilation. Similarly, lactic acid produced during anaerobic respiration also causes vasodilation through its effect on local pH.

Sympathetic nervous system-mediated control of vascular tone

The SNS importantly contributes to the regulation of vascular tone in central arteries, including the carotid and coronary arteries. Feigl et al (1975) demonstrated that, in the absence of myocardial metabolism, a significant change was present in coronary sinus oxygen tension during sympathetic stimulation (84). The contribution of adrenergic receptors in mediating coronary perfusion was subsequently confirmed by Mohrman et al (1978) who found that α-adrenoreceptor blockade caused a decrease in coronary oxygen uptake (85). In a study in cardiac transplant patients, both denervation and regional re-innervation of cardiac segments were studied simultaneously. They demonstrated increases in coronary flow in response to sympathetic stimulation were larger in re-innervated segments compared to denervated segments (86). This finding suggests that coronary flow is regulated to a greater extent by adrenergic mechanisms than via metabolic regulation during sympathetic stimulation. This is key to our study as the carotid artery response is a predominantly NO-mediated mechanism (15, 87). It is challenging to study these factors independently, therefore it remains unclear to what extent

the metabolic and adrenergic components interact and contribute to coronary vasomotion during sympathetic activation (88)

Coronary responses to sympathetic stimulation are highly dependent on both the integrity (89-91) and function (92) of the endothelium. The importance of the endothelium in this response is in part explained through the presence of adrenoreceptors on both the endothelium and SMC. These respond to regional and systemic sympathetic stimuli. For example, local release of norepinephrine from adrenergic nerve terminals in the coronary arteries (abluminal release) and release of catecholamines from the adrenal glands into the circulation during sympathetic stress or physical exercise. These all influence coronary vasomotion through stimulation of adrenergic receptors (93). Both α- and β-adrenergic receptors are involved in coronary vasomotion in response to activation of the SNS (88). Whilst β-receptors induce vasodilation, activation of the α-receptors concurrently induces vasoconstriction (***Figure* 4**). In summary, differences in adrenoreceptor subtypes are present, which ultimately contribute to a delicate balance between α-mediated vasoconstriction and β-mediated dilation.

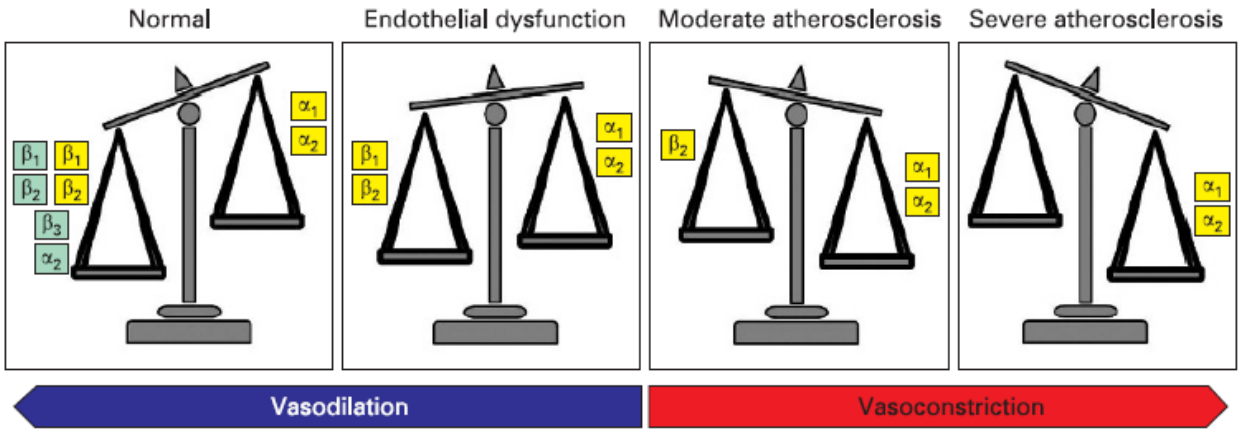


***Figure 4*)** Balance in adrenergic receptors on the endothelium and vascular smooth muscle cells of central arteries. Yellow boxes are endothelium-bound whereas green boxes are SMC-bound. Derived from (88) with permission.

During sympathetic stimulation, healthy endothelium mediates vasodilation through the dilator effects of the endothelium-bound β_1-, β_2-, β_3- and α_2-receptors (green boxes), but also through the β_1- and β_2-receptors on the SMCs (yellow boxes). These dilator effects oppose the constrictor effects of the α_1- and α_2-receptors on the SMCs. In progression of atherosclerosis, loss of endothelium-bound adrenoreceptors contribute to a truncated dilator response and eventually paradoxical constriction (14, 87). Below, these individual receptors are discussed in relation to the regulation of coronary artery vascular tone during sympathetic stimulation.

α1-adrenoreceptors. On the vSMC's, α_1-adrenoreceptors have been identified, which are typically activated through local noradrenaline release from the adrenergic nerve terminals during sympathetic stimulation. Stimulation of α_1-receptors leads to vasoconstriction of coronary arteries (88, 94, 95). Indeed, infusion of α_1-agonists in patients with coronary stenosis demonstrated augmented coronary artery vasoconstriction (88, 90, 91). However, infusion of α_1-agonists in healthy individuals does not alter coronary vascular tone (96). The absence of a vasoconstrictor response in healthy individuals is most likely the result of simultaneous (mild) activation of β-receptors on the endothelium that counterbalances the α_1-mediated constriction.

α2-adrenoreceptors. In addition to stimulation of α_1-receptors, stimulation of the α_2-receptors on the vSMC's mediate neurally-mediated constriction. However, stimulation of α_2-adrenoreceptors located on the endothelium cause dilation through NO-release (88, 96-98). Indeed, several studies using selective stimulation found that α_2-adrenergic agonists cause endothelium-dependent relaxation in coronary arteries of healthy individuals (99), whilst concurrent stimulation of α_2-receptors located on the SMCs mediate coronary artery constriction (96, 98).

Interestingly, intracoronary infusion of selective α_2-agonists induced a paradoxical vasoconstriction in patients with atherosclerotic coronary arteries. This observation is likely explained through the loss of endothelial α_2-receptors in atherosclerotic coronary arteries, which under physiological conditions mediate coronary artery vasodilation. Consequently, α_2-agonists bind to the α_2-receptors located on the SMCs, facilitating coronary artery vasoconstriction. Therefore, the overall effect of α_2-receptor stimulation depends on the net result of both the endothelium- and SMC-located receptors and the functional integrity of the endothelium (96, 100-102).

β-receptors: The β-receptors are predominantly present on the coronary endothelium, causing vasodilation during sympathetic activation. There are 3 subtypes (β_1, β_2, β_3), located on either the endothelium or the vascular SMCs (***Figure 4***), with the β_2-receptor being the most abundantly present and most frequently studied. In healthy participants, infusion of a β_2-receptor agonist (salbutamol) induces coronary vasodilation, suggesting a role for β_2-receptors to lower resistance. Interestingly, administration of propranolol (a non-selective β-receptor agonist), partly inhibited the coronary artery vasodilator responses to sympathetic stimulation (89). Since β_2-receptors are more strongly antagonized by propranolol than β_1-receptors, the β_1-receptor activation contributed to the remaining dilation in healthy individuals (87). In individuals with coronary artery disease, sympathetic stimulation leads to a paradoxical vasoconstriction during sympathetic stimulation. This vasomotor response may result from impaired β-receptor activation. To support this, intracoronary infusion of a β_2-receptor agonist (salbutamol) resulted in impaired vasodilator responses in atherosclerotic coronary arteries (89). This supports a role for β-receptors to contribute to coronary artery vasodilator responses.

Taken together, this highlights the complex antagonistic relationship of both dilatory and constrictive stimuli mediated by α- β-adrenoreceptors on the endothelium and tuna media. This also highlights the complex progression from endothelial function, through to dysfunction and eventual atherosclerosis. It also highlights the loss of expression of adrenoreceptors which may result in this progressive loss-of-function phenotype. This complex relationship is summarized in ***Figure 4***.

Dissimilarities in carotid and coronary artery blood flow and circulation

Although there are significant similarities in both the structure and function in the carotid and coronary arteries, there are also a number of dissimilarities. First, whilst both arteries are similar in structure and function, they experience different flow states. In the coronary arteries, the flow is predominantly diastolic and therefore relatively low pressures, whereas the carotid arteries experience predominantly systolic pulsatile pressure (103, 104). Second, the coronary arteries develop collateral vessel development and microcirculation particularly associated with ischemia. This alters flow patterns within the arteries. Although there is development of collateral micro-vessels within the carotid arteries this is less common than in the coronary arteries (104-106). Third, whilst both the carotid and coronary arteries are equally affected by the endocrine effects of adipokines, the coronary arteries are surrounded by perivascular adipose tissues. This has been demonstrated to have profound endocrine effects on vascular diameter, endothelial function and cardiovascular risk adiponectin as a link between type 2 diabetes and vascular NADPH oxidase activity in the human arterial wall: the regulatory role of perivascular adipose tissue(104, 107, 108).

2.5. Coronary Artery Endothelial Function

The importance of coronary artery endothelial function has been demonstrated previously. Schachinger et al (2000) first demonstrated the importance of coronary artery endothelial function demonstrating that coronary artery endothelial function predicts future CVD in asymptomatic individuals at an increased risk of CVD (42). They investigated coronary artery reactivity to AChand the cold pressor test in 147 patients at an increased risk of CVD. They found patients who suffered cardiovascular events during follow-up had a significantly increased vasoconstrictor response to ACh($p=0.009$) and the CPT ($p=0.002$). Similarly, Rubenfire et al (2000) investigated carotid artery response in 93 participants at average or increased risk of CVD. They used the cold pressor test to investigate carotid artery diameter change using carotid artery ultrasound. They found participants at moderate risk demonstrate carotid artery dilation in response to CPT. An attenuated dilator response in those at an increased risk of CVD and paradoxical vasoconstriction in those with coronary artery disease (14). This was a key finding in investigating carotid artery endothelial function. They termed the change in carotid artery diameter the "Carotid Artery Reactivity".

Carotid artery reactivity (CAR%)

Until recently, ultrasound was typically used to examine static (i.e. IMT) or dynamic (i.e. compliance) characteristics of the carotid artery rather than functional responses to vasoactive stimuli. Based on the earlier description of marked, but similar responses of both the carotid and coronary arteries to sympathetic stimulation. Carotid Artery Reactivity (CAR%) was introduced as an alternative non-invasive method to investigate coronary artery endothelial function (42, 109).

The CAR% refers to the assessment of the carotid artery diameter in response to stimulation of the SNS, using the cold pressor test (CPT). This test involves submersion of a limb in water at a predesignated (typically low) temperature designed to cause a SNS response. The CPT causes stimulation of the SNS, leading to release of norepinephrine and dopamine that contributes to peripheral vasoconstriction, dilation of the carotid and coronary artery and an increase in heart rate (110). It has been demonstrated previously that the CPT in the carotid artery mirrors that of the response in the coronary artery and, therefore that the carotid artery may be used as a surrogate measure of coronary artery responses in healthy individuals (111). The CAR% may be relevant in CAD patients as well as those with increased risk such of CVD as those with CVD risk factors or advanced age. Previous work found that CAD patients demonstrate paradoxical constriction of the coronary and carotid arteries in response to CPT (109).

In addition to the link between CAR% and coronary artery vascular function, recently work has explored the mechanisms underlying responses in the carotid and coronary arteries. The majority of the literature examining the role of adrenoreceptors in mediating central artery responses was performed in coronary arteries. Recently, Van Mil and colleagues examined common carotid (duplex ultrasound) and left anterior descending (LAD) coronary artery (Doppler ultrasound) responses to sympathetic stimulation (using the cold pressor test and lower body negative pressure respectively). These were investigated both with and without α_1-receptor blockade (112). They found carotid and coronary artery dilation during the cold pressor test, with α_1-receptor blockade, lead to an attenuated dilator response in both arteries. In contrast, carotid and coronary arteries showed constriction to lower body negative pressure was not affected by α_1-receptor blockade. Although further work is required, this indicates similarities between

carotid and coronary responses to sympathetic tests (with the direction of these responses being dependent on the type of sympathetic stimulation), as well as in the involvement of α_1-receptors mediation in the responses. In addition, the same researchers demonstrated moderate/strong correlation between the carotid and coronary responses to the CPT (113). This suggests that the CAR% may be used as a surrogate for coronary artery function. Taken together, this suggests CAR% may have a benefit in clinical risk calculators to predict risk of cardiovascular events in patients with pre-existing CAD. As well as in patients at increased risk with no evidence of previous CAD.

Recently, Van Mil *et al.* (2018) found that peripheral arterial disease (PAD) patients who demonstrate carotid artery constriction during the CPT have an increased risk at 1 year of developing cardiovascular events (4.1 times), clinical deterioration (2.0 times) and other adverse events (AEs) (1.8 times) (114). The likelihood of this may also increase with age. This highlights the potential of the CAR% in risk prediction in subjects with increased CAD risk.

Taken together, the literature has shown that both invasive and non-invasive measures of endothelial function have been demonstrated to predict risk in a variety of cardiovascular disease groups. Van mil et al (2018) have demonstrated a correlation between carotid and coronary artery endothelial function (112). They have further demonstrated that carotid artery reactivity predicts risk in peripheral arterial disease patients (54). This thesis aims first, to better understand the mechanisms regulating CAR% and second, to apply the CAR% test to central arterial disease patient, undergoing CABG surgery to investigate if this predicts risk of AEs in central arterial disease patients.

3. Chapter 3: Thesis Goals and Objectives

3.1. Thesis aims

The central aim of this thesis was to investigate CAR% as a method to investigate endothelial function as a surrogate of vascular health This thesis can be divided into two distinct but inter-related studies: First, a study which explored the mechanism's driving CAR% and second, a clinical study which investigated if CAR% can predict AEs and survival in patients undergoing isolated CABG or CABG combined with Valve Surgery.

4. Chapter 4: Methods

4.1. Chapter introduction

This chapter describes the methodology employed in undertaking this research. This includes the ethical application process, SNS provocation using the CPT, diameter measurement and subsequent statistical analysis undertaken. This also highlights if the methodology is utilized in an individual study or both studies.

4.2. Ethical Application

Gaining Ethical Approval

Study one

Ethical approval was gained from University Research Ethics Committee (17/SPS/038).

Study two

Ethical approval for study one was obtained through the National Health Service (NHS) Ethics process (17/NW/0347). This was reviewed by the North West Coast Clinical Research Network and approved on 28/06/2017. No amendments were required.

4.3. Participant Preparation

In preparation for both study 1 and study 2, participants were asked to abstain from smoking for at least 6 hours, from vigorous exercise for at least 24 hours prior to testing and to avoid dietary products that can influence endothelial function, such as caffeine, alcohol, chocolate, and vitamin

C for at least 18 hours (43, 115). Testing was undertaken in a light- and temperature- controlled room. In either a research laboratory (study one) or clinical environment (study two).

4.4. Participant Measurements

Beat-To-Beat Blood Pressure Measurement

In study one, the beat-to-beat blood pressure was measured non-invasively using photoplethysmography (Portapres, Finapress Medical Systems. Amsterdam, Netherlands). An appropriately sized finger cuff was attached to the second phalanx of the right index or middle finger, the transducer was placed on the wrist and a blood pressure cuff was placed on the upper arm. This is demonstrated in figure 5. The finometer was calibrated to the height of the participants heart and was allowed to auto-calibrate for two minutes as per Finapress guidelines (116). Before recording began, the auto-calibration was turned off as this can produce artifacts. The finometer was used to measure relative change in blood pressure throughout the cold pressor test. The reliability and reproducibility of the Finometer Pro has previously been investigated. It has been demonstrated to have excellent reliability and reproducibility as a measure of beat-to-beat blood pressure both at rest and throughout SNS stimulation. This has been demonstrated in both healthy and clinical groups (117-119). This made the Finometer Pro a valuable technique for measuring BP in this study.

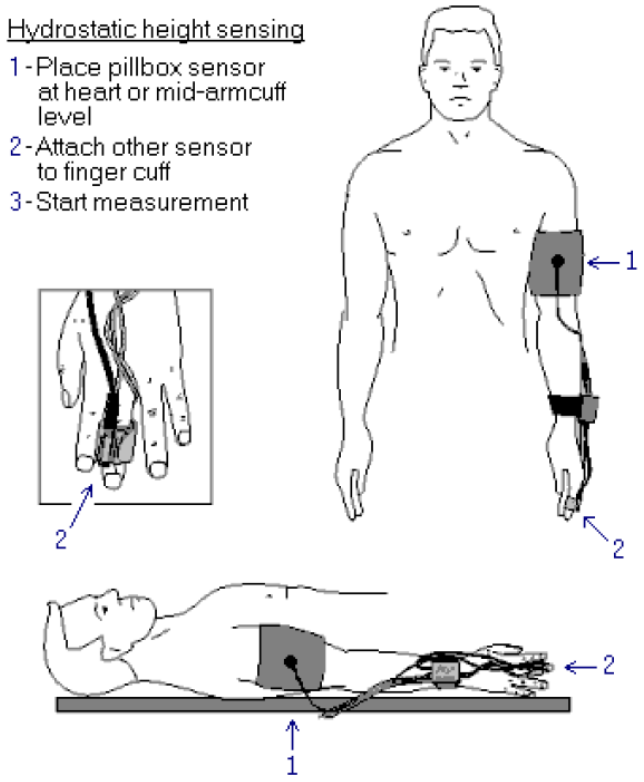


***Figure* 5**) A diagram of correct use and placement of the arm and finger cuff when using Finometer Pro. Derived from (116) with permission.

Blood Pressure Measurement

In study one, the blood pressure was measured with an automated sphygmomanometer (120) on the left arm whilst the participant was laying supine. This was measured twice with a 5-minute break in between. This measure was used to determine the resting blood pressure (BP) and to calibrate the beat-to-beat blood pressure values. Automated Sphygmomanometer has been previously and extensively validated for accurate measurement of resting BP (121)

Cold pressor test

In both study one and study two, the CPT was used as an SNS stimulus. During the CPT, the left hand was immersed in a bucket of cold water (~4°C). The water temperature was measured with a digital thermometer (Quartz digi-thermo, Fischer scientific, Loughborough, UK) and controlled by adding crushed ice to maintain a stable water temperature. The participants were asked to position themselves close to the left edge of the bed, to ensure the hand could easily move into the water without significant movement of the neck. This enabled assessment of the carotid artery. After a 1-minute baseline period, participants were instructed to place their hand in the ice water for a predesignated time period. This is specified within the methods section of each study. The CPT has previously been demonstrated to have good same-day and next-day reliability and reproducibility (115, 122). The CPT has also been demonstrated to be safe in a variety of healthy and clinical populations (14). The arterial response in both healthy participants and in those at risk of CVD has been shown to be similar in response to acetylcholine (gold standard) and the CPT (109). The CPT was therefore used as a reliable, reproducible and safe technique used to stimulate the SNS. Participants were instructed not to speak, and to breathe normally in order to prevent hyperventilation. Hyperventilation has been demonstrated to alter carotid artery diameter (54, 115, 123).

Carotid Artery Diameter Analysis

The left common carotid artery (CCA) diameter was assessed using ultrasound sonography (L9-3 MHz linear array probe attached to a high-resolution ultrasound machine (Terason 3300, Terason Labs, Burlington, Massachusetts, USA)). A longitudinal view of the artery was first used to identify the carotid bulb. This was identified as an anatomical landmark to standardise approximate

scanning area between individuals. A segment of approximately 2 cms from the bifurcation was used to avoid turbulent flow. The common carotid artery, proximal from the carotid bulb, was identified and image was optimised using time gain compensation and tissue depth settings. This was used to ensure vessel walls are clearly defined with maximum contrast between vessel walls and surrounding tissue (***Figure 6***). Doppler velocity assessments were also recorded at the lowest possible angle of insonation (always <60°). The carotid artery diameter was calculated with edge detection software (124). On-screen calibration points were selected with the calibration tool. This is used to calibrate pixel-to-centimetre ratio. Calibration points were used for the diameter and the pulse wave velocity. A rectangle containing the largest straight artery segment was selected as the Region of Interest (ROI), ensuring that the vessel walls were in focus.

The wall-tracking software marked the vessel walls within the ROI. The ROI used is approximately one third the length of the entire segment analysed. The number of pixels in each vertical column between vessel walls are calculated automatically. This was then used to calculate the lumen diameter in centimetres. This has been used previously and has been demonstrated to be reliably and independent of user influence (49, 111). All analysis was performed blinded and separately by two independent researchers (125). The sensitivity and specificity of carotid artery ultrasound in both research and clinical medicine has been extensively researched and demonstrated previously (115). Carotid Artery Ultrasound remains an accurate, reliable and reproducible non-invasive method to investigate both static and dynamic carotid artery diameter (126-129)

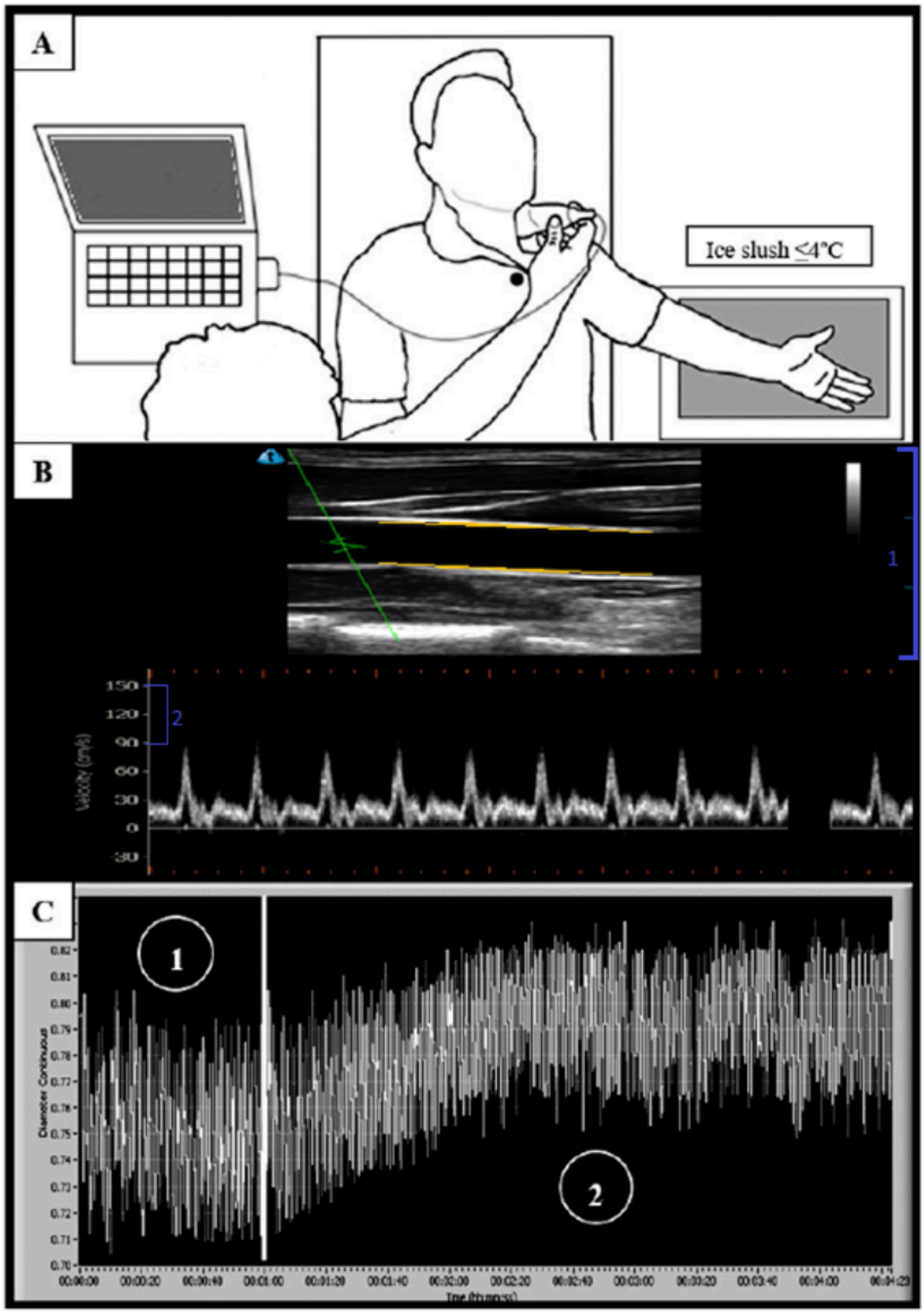


***Figure* 6**) A panel demonstrating Carotid Artery Response (CAR) measurement. **A**) A schematic representation of carotid artery response setup demonstrating imaging of the carotid artery

whilst the participants hand is in icy-cold water at 4°C. **B**) An image of a participants carotid artery demonstrating the wall tracking software and pulse-wave measurement including standardization tools for **1:** standardization for diameter measurement and **2:** standardization for pulse-wave velocity calculation and **C**) Demonstrates the carotid artery response to the cold pressor test including **1**: Baseline diameter recording and **2**: Whilst the hand is submersed in icy-cold water. Adapted from (54, 130)

Carotid Artery Response (CAR%)

The CAR% is the relative change in carotid artery diameter above or below baseline expressed as a percentage. The average diameter during the 1-minute baseline measurement was calculated and used as the baseline value. Subsequently, the diameter of the carotid artery was measured during the CPT and averaged over 10 second periods. The average, maximum and minimum percentages, were calculated. If the average percentage change was an increase in diameter (dilation), the CAR% was expressed as the maximum percentage. Conversely, if the average percentage change was a decrease in diameter (constriction), the CAR% was expressed as the minimum percentage. This is in-line with previously published work (54, 115, 123).

Continuous BP Image Processing and Statistical Analysis

Continuous BP analysis was undertaken for study 1. However, this could not be replicated for study two, as the equipment is not transferable to the hospital setting. This would have been beneficial to include in both studies as an increase in blood pressure may play a part in the dilatation/constriction response in response to SNS stimulation.

The CAR% was calculated as described above. The BP of each participant was recorded continuously using the finometer (116). The baseline was calculated as the average of the BP

recordings across the 1-minute baseline measurement. The averaged BP measurement was then calculated for each ten second period. This was then correlated with carotid artery diameter to investigate if BP was correlated with CAR%. The mean BP was also correlated with CAR% to investigate the association of BP (dependent upon peripheral atrial endothelial function) and CAR% (central arterial endothelial function).

Considerations in vascular ultrasound

Carotid artery ultrasound is used commonly in clinical practice and has been demonstrated to have excellent same-day and next-day reliability (115). However, there are still considerations associated with the use and interpretation of ultrasound.

First, although ultrasound has been demonstrated to have excellent reproducibility, this is highly practitioner dependent in both scan acquisition and in analysis. All participants in both of these studies were scanned on one occasion and therefore the reproducibility and reliability could not be investigated. In future studies and in clinical practice it is key to demonstrate that individual researchers and practitioners are suitably competent. This was unfeasible in this study.

Second, whilst it has been shown to be reliable and reproducible, ultrasound is highly dependent upon proper technique and standardised protocols. The area proximal to the carotid bifurcation experiences turbulent flow and this may affect vasomotion and therefore CAR%. A segment 2-3cm from the carotid bulb was therefore used to avoid this affecting recording. This was standardised throughout all participants. Similarly, researchers have to select an appropriate Region of interest (ROI) to use as a representative sample of the entire vessel. If a ROI is used

that is too small it may not be representative of the entire vessel. A ROI was therefore always used which is approximately one third of the entire length of segment scanned.

Taken together, although vascular ultrasound is a commonly utilized, accurate and reliable technique. It is highly user-dependent. In future studies, researchers should demonstrate competency before undertaking large-scale clinical trials.

Calculating QRISK3 score

The QRisk3 score is a score of the probability of developing CVD in individuals with no history of CVD (131). The QRISK score was calculated using participant demographic data (age, weight and smoking status) as well as medical history. This was then used to calculate QRISK using an online calculator.

4.5. Risks and Considerations during this study

There were a number of risks and potential implications associated with both of these studies. First, previous studies have suggested that the cold pressor test may cause angina chest pain in some patients (132, 133). This chest pain was relieved by removing the hand from the water (132, 133). Participants were therefore instructed to remove the hand from the water if they feel any discomfort. In study one, this research was undertaken in a busy research lab with first aid-trained staff available. The risk of chest pain associated with the CPT was higher in patients with established cardiovascular disease. In study 2, these participants therefore underwent testing in a clinical environment. A number of the techniques employed in this PhD are also used clinically. There was a risk of identifying potentially clinically significant incidental findings. If any incidental

findings were identified, the participant were referred to a relevant clinician – either their GP (study one) or consultant physician (study two).

4.6. Data Analysis and Measurements

All data is first examined for the "level" of data. The kurtosis and skewness was measured using a D' Agostino-Pearson goodness-of-fit test. If there were two variables and the data was normality distributed, a t test was used. If the data was non-normally distributed, the non-parametric equivalent was used. If there were more than two variables, a one-way ANOVA was used. If the data was non-normally distributed, the non-parametric equivalent was used. If the data was binary, a Chi-squared and Fischers exact test were used (95% confidence interval). Whereas the Chi-squared test assumes a large dataset and therefore provides an approximation, the fischers exact test provides an exact value and is therefore more powerful in datasets with less than five counts (134). If the data was continuous, normality testing was first performed using D' Agostino-Pearson goodness-of-fit testing. A Pearson correlation was used is the data was normally distributed. If it was non-normally distributed, a Spearman correlation was used. Statistical significance is defined as a $P<0.05$. The study-specific statistical methodology is discussed within the "methods" section of the study chapter.

5. Chapter 5: Study One - A Mechanistic Study of CAR%

5.1. Chapter introduction:

This chapter describes a pilot study investigating the relationship between blood pressure and CAR%. This study aims to investigate the degree to which blood pressure response drives CAR%.

5.2. Research question:

Is resting blood pressure related to the magnitude of carotid artery responsiveness and blood pressure changes during the CAR% in middle-aged healthy volunteers?

5.3. Aim:

To investigate the relationship between resting blood pressure (a marker of peripheral arterial endothelial dysfunction) and carotid artery diameter and blood pressure responses during CAR% test.

5.4. Objectives

1. **Does endothelial function relate to increase in blood pressure in middle-aged healthy men?**

   To investigate whether CAR% relates to the blood pressure increase during the CAR-test in middle-aged healthy men.

5.5. Hypothesis

H_1 The increase in blood pressure during the CAR% is related to baseline blood pressure and/or the magnitude of carotid artery diameter change.

5.6. Abstract

Carotid artery diameter responses to sympathetic stimulation measured using carotid artery reactivity (CAR%) represents a novel test of vascular health and relates to cardiovascular disease risk. However, the regulation of CAR% is incompletely understood. This study aimed to investigate the relationship between the increase in blood pressure and carotid artery diameter response during the CAR-test in healthy, middle-aged men. The study sample consisted of 40 normotensive men (aged 31-59) with no history of cardiovascular disease or currently taking medication. Non-invasive ultrasound was used to measure carotid artery diameter during the cold pressor test (CPT). The CAR% being calculated as the relative change in carotid artery diameter from baseline and expressed as a percentage. Mean arterial pressure (MAP) was measured with beat-to-beat blood pressure recording. The average CAR% was 4.4±5.4%, peaking at 92±43s. MAP increased from 88±9 mmHg to 110±15 mmHg, peaking at 112±38 seconds. Peak MAP was significantly later than the diameter peak (P=0.04). The correlation between resting MAP and CAR% was weak (r=0.209 P=0.197). Tertiles based on resting MAP or MAP-increase revealed no significant differences between groups in subject characteristics including age, BMI or CAR% (all P>0.05). Subgroup analysis of individuals with carotid constriction (n=6) versus dilation (n=34), revealed no significant difference in resting MAP or increase in MAP (P=0.209 and 0.272, respectively). Taken together, the data presented in this study suggested that the characteristic increase in MAP during the CPT did not significantly drive the carotid artery response.

5.7. Background

The vascular endothelium plays an important role in regulating vascular tone, thereby contributing to the health and integrity of the vasculature. Several studies have revealed the importance of a healthy endothelium in the prevention of the progression of atherosclerosis and development of cardiovascular disease (15). Endothelial function has been demonstrated to predict cardiovascular disease in asymptomatic individuals, disease progression in patients with established cardiovascular disease as well as AEs and survival in patients with established CAD undergoing treatment (38-41).

It has been previously demonstrated that participants with established cardiovascular disease demonstrate paradoxical constriction in response to SNS stimulation (109). Vasoconstriction in central vessels in response to SNS stimulus has been demonstrated to predict a higher risk of the development of CVD in asymptomatic individuals (135, 136) and disease progression and survival in patients undergoing treatment for CVD (14, 42, 137). A variety of techniques investigated vasomotion as surrogates of endothelial function. The regulation of vascular tone is achieved through a complex inter-connected system. This was discussed in brief below but discussed in greater detail in the "*Background"* chapter of this thesis.

Mechanisms of vascular tone regulation and shear stress

Vasomotion is not the result of endothelial function alone but is a response regulated by a complex system which matches perfusion with requirement. This was reviewed in the *"Background"* chapter of this thesis. This is particularly important when investigating vasomotion in the context of provocative tests including the cold pressor test. If endothelial function, measured through vasomotion is to be used clinically, It was key to try to delineate the degree to which endothelial function regulates vasomotion. Although the CAR% method investigates vasomotion in the carotid artery, due to the inability to properly isolate the carotid artery, the coronary arteries are more commonly used within the literature. The direct measurement of coronary artery endothelial function requires invasive measurement and is therefore costly and outside of the remit of this PhD. Although this has been discussed more thoroughly in the "*background*" chapter, this was discussed in brief below. The regulation of vascular tone is predominantly achieved through 3 mechanisms.

Metabolic regulation of vascular tone

The majority of research investigating the regulation of vascular tone through metabolic demand has been investigated within the coronary arteries. Therefore, the research discussed below was predominantly conducted in the coronary arteries. It has been demonstrated previously that change in metabolic demand within the myocardium produce profound changes in myocardial perfusion. This in-turn results in change in coronary artery vascular tone.

Krivokapich et al (1989) investigated myocardial perfusion using 13N ammonia myocardial imaging with Positron Emission Tomography in healthy participants at rest and throughout a

supine bicycle exercise test. They demonstrated that a 2.8-fold elevation in myocardial work (demand) was matched by a 2.2-fold increase in myocardial perfusion. Similarly, and importantly, the same group investigated myocardial demand and perfusion in both healthy volunteers and those with established coronary artery disease, demonstrated through angiography. They found healthy participants demonstrated a significant increase in perfusion throughout the supine bike exercise. Whereas patients with significant coronary artery disease demonstrated a myocardial requirement-to-perfusion mismatch (138). Taken together this demonstrated first, that metabolic demand was a key stimulus resulting in significant increases in myocardial perfusion through increase in perfusion and second, that flow-limiting coronary artery disease hampers the ability to increase myocardial perfusion. This was thought to be caused by changes in coronary artery diameter as a result of CAD. Although this was not measured by Krivokapich et al (1989).

Neurally-Mediated vascular tone

The SNS plays a key role in maintaining and regulating vascular tone in both peripheral and central arteries. The vascular tone is the degree to which vasoconstrictive and vasodilatory stimuli affect diameter thereby allowing an increase or decrease in diameter of the vessel in response to physiological stimuli (14). This is largely thought to be regulated through α- and β-adrenergic receptors on the endothelium and tunica media (87). It has been demonstrated previously that SNS stimulation leads to marked vasodilation within the central arteries including the coronary arteries (42). However in the context of progressive loss of endothelial function, through endothelial dysfunction and eventual atherosclerosis, it is thought that there is an alteration in endothelial- and SMC-bound α- and β-adrenoreceptor expression (87). In health

there is a balance between the pro-dilatory effects of the endothelium-bound β1, β2-, and β3- and α2- adrenoreceptors and the SMC-bound β1- and β2-adrenoreceptors acting in opposition to the pro-constrictive effects of SMC bound α1- and α2- adrenoreceptors (88). This is summarized in ***Figure 4*** in the "Background" section of this thesis. It is thought that, with the development of endothelial dysfunction and eventual atherosclerosis, there is a loss of expression of pro-dilatory adrenoreceptors resulting firstly in a truncated dilatation and eventually in a constriction in response to an SNS stimulus (14). Mohrman et al (2013) investigated coronary oxygen uptake alongside α-adrenoreceptor blockade in the left anterior descending artery of healthy participants throughout the cold pressor test (86, 139). They found reduced oxygen uptake with α-adrenoreceptor blockade. This highlights the importance of neurally-mediated vasomotion.

In healthy individuals, there is a net dilatory effect on both the coronary and carotid artery circulation in response to an SNS stimulus. Whereas in patients with coronary artery disease, sympathetic stimulation leads to a paradoxical vasoconstriction during sympathetic stimulation (42, 65). This may be as a result of impaired β-adrenoreceptor activation. In support of this, the β-adrenoreceptor agonist Salbutamol produces impaired vasodilator response in patients with established atherosclerotic coronary artery disease (14). Recently, Van Mil et al (2018) investigated carotid and coronary artery responses to sympathetic stimuli alongside α_1-adrenoreceptor blockade. They found attenuated carotid and coronary artery dilation in response to the cold pressor test alongside α_1-adrenoreceptor blockade. In contrast to this, the coronary and carotid arteries constrict in response to lower body negative pressure both with and without α_1-adrenoreceptor blockade. Lower body negative pressure is a chamber at the level

of the iliac crest whereby pressure is reduced in -10mmHg increments until it reaches -80 mmHg or until pre-syncope. This is used as an SNS stimulus. Taken together, this demonstrates first that the carotid and coronary arteries demonstrate similarities in responses to sympathetic stimulation and second that α_1-adrenoreceptors are key in mediating this response.

The extent to which individual mechanisms regulate vascular tone is key to investigating vasomotion as a surrogate of endothelial function. Vasomotion was investigated in denervated and regional re-innervated coronary artery segments of patients who had undergone cardiac transplantation (14, 88). It was found that coronary flow response to sympathetic stimulation is greater in re-innervated segments when compared to denervated segments (14, 88). This may suggest that the increase in coronary flow response to sympathetic stimulation is achieved to a larger extent through neurally-mediated rather than metabolic-mediated mechanisms. Although investigating the interaction between the metabolic and adrenergic components is very challenging and therefore, this is not completely understood.

Shear Stress -Mediated vascular tone

Endothelial function has a key role in vascular tone. However, shear stress (SS) has been demonstrated to have a profound role in regulating vascular tone. SS is the force that blood has on the endothelium as it passes through the vasculature. This friction force is opposed by tension and deformation in the endothelium, whilst the circumferential distension of blood pressure is opposed by circumferential stress (stretch) in the vessel wall (14). SS has been demonstrated to have a profound endothelium-dependent dilatory effect on arteries in both a dog ileac artery model and in human arteries including the carotid and brachial arteries (14, 140, 141). The effect

of SS on carotid artery diameter is particularly important in this study. This SS-mediated dilation is achieved through stretch mechanosensors and is dependent upon the magnitude and direction of flow and the activity of NADPH oxidase. The sensing of changes in shear stress is achieved through several pathways such as platelet endothelial cell adhesion molecule 1 (PECAM-1), integrins, ion channels and tyrosine kinase receptors (142). It was demonstrated that NO inhibition abolished SS-related dilatation demonstrating that SS-mediated dilatation was a function of endothelial function through NO production (91, 143). This therefore means that an increase in blood pressure may affect vasomotion through the SS stimulus and that the magnitude of blood pressure increases and therefore increase in SS may impact CAR% (91, 143). Vita et al (1992) investigated coronary artery SS in healthy and atherosclerotic coronary arteries by increasing coronary artery blood flow using infusion of intracoronary adenosine. They found that, in healthy coronary arteries, increase in SS results in a coronary artery vasodilation (91, 143). This is likely through the increase in transmural pressure. Whereas in atherosclerosis, failure of this dilatory mechanisms results in greater change in SS which may negatively affect expression of anti-atherosclerotic transcription factors (144). This is achieved through an alteration in expression patterns of pro- and anti-atherosclerotic genes, tipping the balance towards pro-inflammatory and pro-atherogenic associated with turbulent flow. This flow-specific response is achieved through endothelial sensing of ROS (144). Similarly and importantly an increase in SS caused by hypercapnia results in an increase in vasodilation of the internal carotid artery in healthy individuals (145). Taken together, this highlights the ability of SS to affect carotid artery diameter.

Shear Stress (SS) and CAR%

Shear stress is relevant when investigating vasomotion as a surrogate of endothelial function because an increase in blood pressure as seen during the CPT may affect vasomotion as a hemodynamic stimulus. The magnitude of blood pressure increase may reflect sympathetic drive (36, 146). This therefore highlights the importance of better understanding the effect of blood pressure (and therefore SS) on endothelial function and therefore vascular tone.

In an attempt to better understand the effect of blood pressure (and therefore SS) on vasomotion. This study investigated the relationship between the timing and magnitude of a sympathetically induced elevation in blood pressure and carotid artery diameter responses in healthy, middle-aged men. This group was included because this group demonstrates a good diversity of blood pressure and diameter responses to the cold pressor test, whilst being less likely to have significant age-related negative impacts on endothelial function. The endothelially-protective effects of estrogen are also a key confounding variable and therefore we investigated men only (77, 147). In future studies, sub-group analysis including pre- and post-menopausal women and women receiving hormone replacement therapy should be investigated. An increase in blood pressure is associated with a relatively large transmural pressure (i.e. hemodynamic stimulus relevant for vasomotion) and sympathetic stimulation (i.e. stimulus for carotid artery vasomotion). This increase in blood pressure will result in an increase in shear stress. It is hypothesised that a large blood pressure change (and therefore shear stress) will produce a larger dilatory stimulus and therefore a larger CAR%. This is a potential confounding variable and therefore this study aims to investigate the degree to which the increase in BP (and therefore SS) drives CAR% in healthy male participants.

5.8. Methods

Participant Recruitment

Forty healthy men aged 31-59 years old with no history of cardiovascular disease were recruited from Liverpool John Moores University. Exclusion criteria were: a history of cardiovascular disease, history of diabetes, currently using cardiac medication for cardiac arrythmias, hypertension or hypercholesterolemia or those who have known or suspected Raynaud's syndrome or those with known or suspected carotid artery disease or endarterectomy. All of which were self-reported through the pre-testing questionnaire. Any participants who described Raynaud's-like symptoms or who weren't sure were also excluded. This exclusion criteria were selected as these are all known to affect endothelial function or blood pressure response (49, 54). As this study was investigating the relationship between endothelial function and blood pressure, participants with confirmed conditions or medication which is known to affect vasomotion, blood pressure response or SNS activation were also excluded. This was in-line with current guidelines for investigating endothelial function (49) and was in-line with previously published research (54).

Procedure

Local ethical approval from the Liverpool John Moores University was gained on 17/8/2017 (17/NW/0347). Informed consent was obtained and formally documented for each participant. Participants were asked to abstain from smoking for at least 6 hours, from vigorous exercise for at least 24 hours prior to attending the laboratory and to avoid dietary products that can influence endothelial function, such as caffeine, alcohol, chocolate, and vitamin C for at least 18

hours. This was in line with current guidelines and previously published work (43, 115). Participant's body weight and height were measured on arrival. Participants would then lay on a bed in a quiet, light and temperature controlled room (43, 115) and completed a non-validated healthcare questionnaire which included medical history, CVD related lifestyle risk factors, family history and current/previous medications prior to testing. The QRisk3 score was calculated for each participant using the online QRisk3 calculator. This is used clinically to predict risk of cardiovascular disease and events in patients with no history of cardiovascular disease (148)

Physiological Measurements

Blood pressure

The resting blood pressure reading was measured with an automated sphygmomanometer (Dynamap Procare 400) in duplicate with 5 minutes in-between (120). A period of at least 5 minutes was allowed after the BP cuff was used before the cold pressor test was performed. This is because inflation of the BP cuff may have an effect on the SNS and therefore alter CPT response. The left arm was used whilst the participant was laying supine. This measure was used to calibrate the beat-to-beat blood pressure values. To measure beat-by-beat blood pressure, a finapress finometer pro model was used. The second phalanx of the right index or middle finger was measured and the most appropriate finometer finger cuff selected. The finometer was calibrated to the height of the heart for each individual participant. The finometer was then allowed to auto-calibrate for two minutes as per manufacturer instructions. The correct participant preparations and finometer cuff place is demonstrated in ***Figure 5.*** This was in-line with manufacturer's guidelines (116). The finometer has been demonstrated to be a reliable and

reproducible measure of beat-to-beat blood pressure monitoring during both resting (117) and throughout SNS stimulation using both the cold pressor test and exercise stimulus (149) .

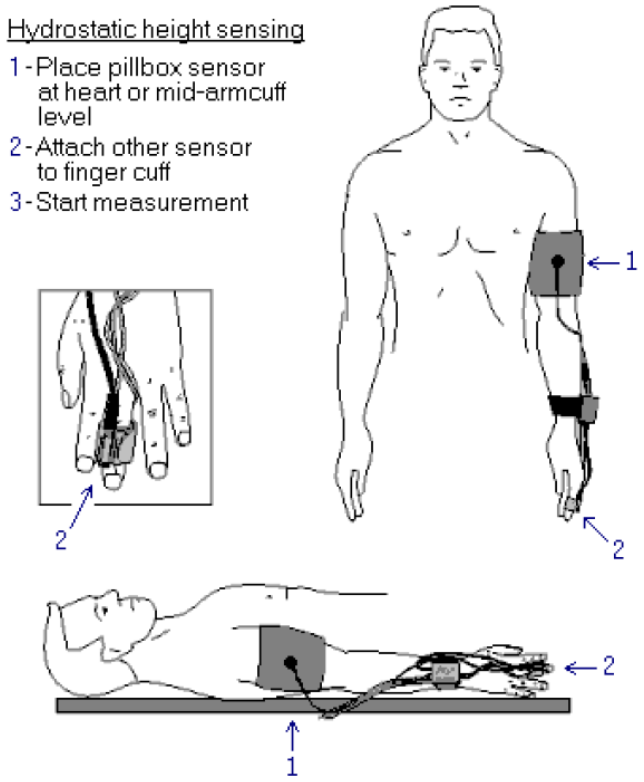


***Figure* 5)** A schematic representation of appropriate arm, finger and wrist placement taken from Portapress Finapress manufacturer guidelines (116) with permission.

Cold Pressor Test

The Cold Pressor Test has been previously demonstrated to be a reliable and reproducible method of stimulating the SNS in both healthy participants and in those with cardiovascular disease (42, 115). During the CPT, the left hand was immersed in a bucket of cold water (~4°C). This temperature has been previously demonstrated to be sufficient to elicit a safe and

reproducible SNS response whilst being well tolerated with participants (54). The water temperature was measured with a digital thermometer (Quartz digi-thermo, Fischer scientific, Loughborough, UK) and controlled by adding crushed ice to maintain a stable water temperature. The participants were asked to position themselves close to the left edge of the bed, to ensure the hand could easily move into the water without significant movement of the neck. This avoided artefacts or movement during recording.

Participants were instructed not to speak, to breathe normally and to remain as still as possible throughout testing. As participants may hyperventilate, they were encouraged throughout. This avoided disturbance throughout recording. This was in-line with previously published research (115, 130). A one minute "baseline" period was first recorded prior to the hand being placed in the water. After the initial one-minute period, the participant was instructed to place the hand in to the water whilst remaining still. The hand was held in the water for three minutes. Participants were also asked not to speak, and to breathe normally throughout to avoid hypercapnia. The participant was verbally encouraged throughout (54, 115, 123).

Carotid Artery Diameter Imaging

The left common carotid artery diameter was imaged using vascular ultrasound sonography (Terason 3300, Terason Labs, Burlington, Massachusetts, USA). The carotid bulb of the left carotid artery was first imaged in a longitudinal view. The carotid bulb was identified as an anatomical landmark and used to standardise approximate scanning area between individuals. A segment proximal from the carotid bulb, was identified and the image was optimised using time-gain compensation and depth. The image was optimised until artery walls were clearly defined

with maximum contrast between vessel walls and surrounding tissue. (***Figure 9***). On-screen calibration points were selected with the calibration tool. This allowed software to calculate the pixel-to-centimetre ratio. The calibration points were used for the diameter and the pulse wave velocity. A rectangle containing the largest straight artery segment was selected as the (ROI), ensuring that the vessel walls were in focus. The software marked the vessel walls within the ROI with lines and calculated the number of pixels in each vertical column. From the pixel distance the software calculated the lumen diameter in centimetres. This was all in line with previously published research (14, 54, 115) and is discussed in further detail in the "*methods*" section of this thesis.

Carotid Artery Response (CAR%)

The CAR% is the relative change in carotid artery diameter above or below baseline expressed as a percentage. The average diameter during the one-minute period before submersion was calculated and used as the baseline value. Subsequently, the diameter of the carotid artery was measured during the CPT and averaged over ten second periods throughout both baseline and throughout the stimulus. The average, maximum and minimum percentages were calculated. If the average percentage change was an increase in diameter (dilation), the CAR% is expressed as the maximum percentage. Conversely, if the average percentage change was a decrease in diameter (constriction), the CAR% is expressed as the minimum percentage. This was in-line with previous work utilizing the cold pressor test. (54, 115, 123)

Blood pressure

The beat-to-beat blood pressure data was processed in the same way as the CAR% calculation. Beat-to-beat blood pressure was measured and then MAP calculated. Baseline was the average MAP (AVGMAP) during the one minute prior to the hand being placed in water. The average MAP during the CPT was calculated for each ten second period, matching the epochs for diameter as described earlier. The systolic and diastolic blood pressure measured with the sphygmomanometer was used to calculate the MAP before testing. The beat-to-beat blood pressure data was calibrated using the resting blood pressure measured using an automated sphygmomanometer (Dynamap). The cuff was placed around the left arm and performed twice (with a five-minute rest period in between). A period of at least 10 minutes was given between this and performing the CPT. The maximum change in blood pressure was expressed as the maximum increase (ΔMAP) and maximum percent increase in MAP (relative ΔMAP) compared to baseline during the CPT.

Statistical analysis

All data were presented as mean ± SD. Statistical analysis was performed using IBM SPSS Statistics 25 (IBM SPSS; IBM Corp., Armonk, New York, USA). Pearson correlations were employed to examine the relationship between baseline MAP and the change in blood pressure during the cold pressor test (ΔMAP) versus the CAR% (i.e. relative change in diameter compared to baseline). The relationship between the timing of the peak responses in BP versus CAR% was also investigated using a Pearson correlation. Participants were divided in to tertiles based on the relative change in BP during the CPT: low (<15%), medium (15-30%) and high (>30%). One-way ANOVA was used to examine difference between groups in general characteristics including age,

BMI and cardiovascular risk and CAR%. Tukey post-hoc analysis was performed to examine which groups differed from each other. Statistical significance was set at $p<0.05$.

5.9. Results

The results were divided in to six different sections. First, a description of the characteristics of the population. Second, a study investigating the change in both carotid artery diameter and blood pressure in the whole group throughout the CPT. Third, a study investigating the effect of age on endothelial function to investigate age as a confounding variable. Fourth, a study investigating the relationship between cardiovascular risk factors, MAP and carotid artery diameter to investigate cardiovascular risk factors as confounding variables. Fifth, the relationship between change is blood pressure throughout the CPT and CAR% when healthy male participants are grouped according to their blood pressure response to the CPT. Sixth, is investigating the demographics in participants grouped according to if they demonstrate a "dilation" or "constriction" CAR% response throughout the CPT. This was to investigate the impacting of confounding variables on the CAR% response in this group.

Participant Demographic Data

40 healthy men aged 31-59 were included. The average age of participants was 46±12 years. These participants had an averaged BMI of 27±4 kh/m^2. They were normotensive with a systolic BP of 123±12 mmHg and a diastolic BP of 74±5 mmHg. The average QRisk3 score was 4.9±4.94. A secondary aim of this study was to investigate endothelial function in both normotensive and hypertensive participants, to investigate if there is a significant difference in central arterial

endothelial function in normotensive and hypertensive participants. However, as all participants were normotensive, this was not possible.

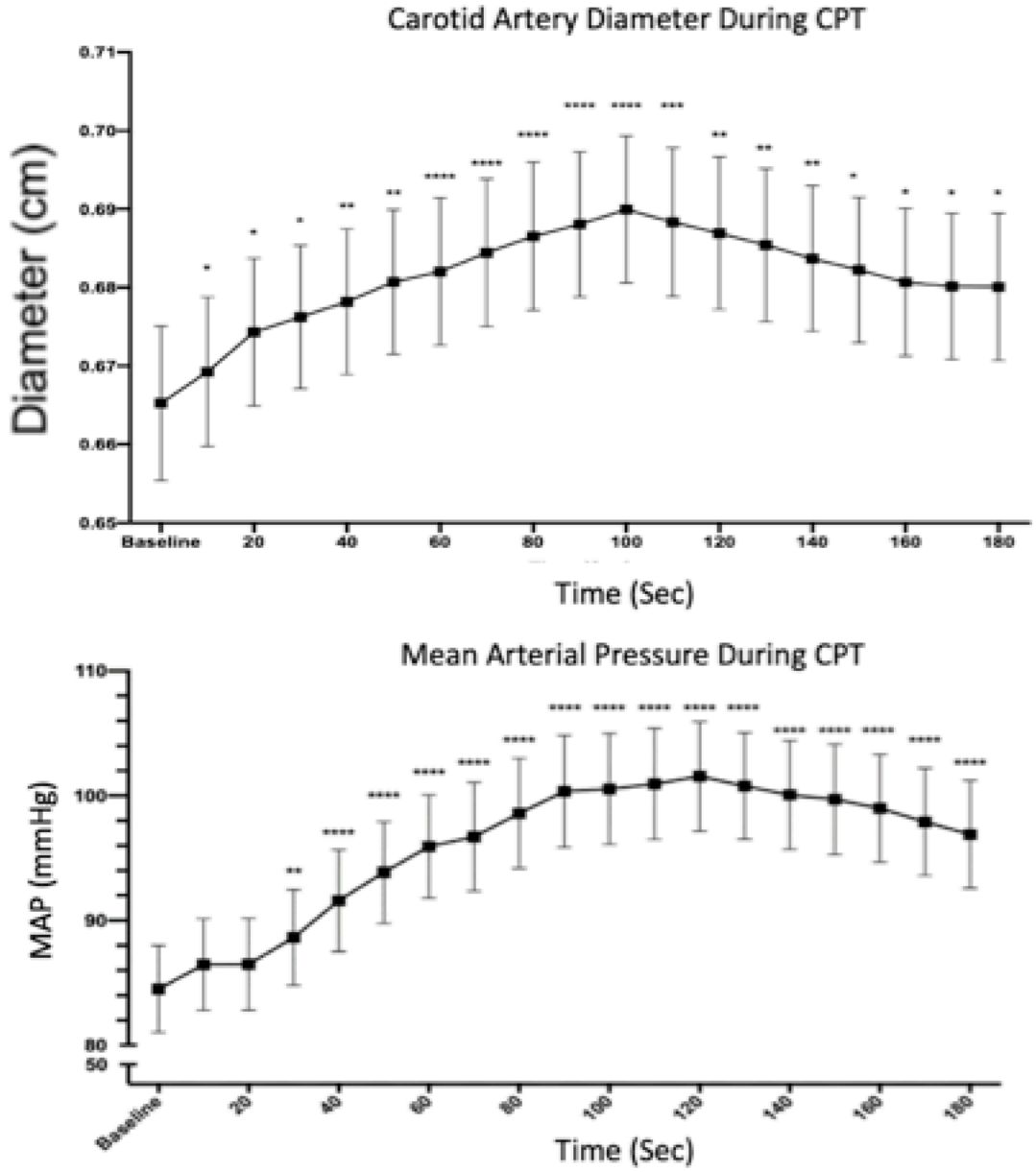
***Table* 2)** Demographic data of all participants involved in the trial. Demographic data is expressed as mean with SD.

Age (years)	46±12
Weight (kg)	80.41±15.32
Height (meters)	1.73±0.05
BMI (kg/m^2)	27±4
Systolic BP (mmHg)	123±12
Diastolic BP (mmHg)	74±5
QRISK (%)	4.91±4.94

Diameter and Blood Pressure Response to the CPT

This study first began by investigating both the change in both carotid artery diameter and brachial artery MAP throughout the CPT in all participants. This also investigated the relationship between timing of peak diameter and peak MAP response. The MAP is derived from beat-by-beat blood pressure recording. The MAP gradually increased with all participants demonstrating a statistically significant increase in MAP throughout the CPT (figure 7). MAP gradually increases within 30 seconds. Peak MAP occurred at 112±38 seconds with a gradual decline at ~130 seconds. There was also a statistically significant increase in mean diameter through the CPT. This is demonstrated in figure 7. The diameter gradually increased after a period of ~10 seconds and peaked at 92±43 seconds. A Pearson correlation was used to investigate the correlation between MAP and diameter. There was no correlation between peak MAP and peak diameter timing in this group ($R = 0.03$, $P = 0.30$).

***Figure 7*)** Mean and Standard Deviation of all-participants response during the cold pressor test (CPT). Both carotid artery diameter and brachial artery blood pressure plotted against time. One-Way ANOVA performed to investigate statistical significance of change from baseline measurement to increase in both MAP and diameter plotted against time. * denotes P= <0.05, ** denotes P:<0.01, *** denotes P=<0.001 ****denotes P:0.0001.



Young vs old participants

To investigate the impact of age on response participants were divided according to their age. "young" participants were considered as those aged 31-45 and "older" patients as 46-60. It is thought that the decline in endothelial function is non-linear and associated with "unhealthy" vs "healthy" aging and significant fall in in "middle aged" participants. The participants were therefore grouped in to "young" and "old" rather than a correlative relationship. here was a statistically significant difference in age between these two groups (P = 0.0001). There was also a statistically significant difference in weight (P=0.0196), BMI (P = 0.0247) and QRisk3 (P= 0.0001). The "older" participants had a higher height, weight, BMI and QRisk3 score (***Table 3***). When examining both MAP and diameter data at baseline and throughout the CPT, there was also a statistically significant difference in average diameter (P = 0.0408) and max diameter (P = 0.0313). When comparing "Young" vs "Old" participants (***Table 4***). The "older" participants had a higher average and maximum diameter throughout CPT.

***Table 3*)** Demographic data of participants separated according to their age into young (n=26) and older (n=14) subjects including SD. An independent samples t test was performed. Significance defined as p=<0.05

	Young (31-45 yrs) (n=25)	Older (46-60 yrs) (n=15)	P Value
Age (years)	36±5	53±5	0.01*
Weight (kg)	78±11	90±13	0.01*
Height (meter)	1.76±0.07	1.78±0.06	0.49
BMI (kg/m^2)	25±3	28±4	0.02*
Systolic BP (mmHg)	122±9	128±14	0.08
Diastolic BP(mmHg)	71±11	72±8	0.51
QRISK (%)	1.52±1.33	6.91±2.82	0.01*

***Table 4*)** Mean Arterial Pressure and carotid artery diameter responses in participants at baseline and average MAP throughout the CPT in participants when separated according to "young" (30-45, n=26) and "older" (46-60, n=14)

	Young (n=25)			Older (n=15)		
	Baseline	**AVG CPT**	**MAX CPT**	**Baseline**	**AVG CPT**	**MAX CPT**
MAP (mmHg)	87±9	99±13	108±14	91±10	104	115±17
Diam (cm)	0.65±0.05	0.66±0.04	0.68±0.04	0.69±0.07	0.71±0.07	0.73±0.07

***Table 5*)** Statistical comparison of "old" vs "young" baseline, average and max MAP and CAR% throughout the CPT. "Young" defined as aged 30-45 (n= 26) and "old" defined as 46-60 (n=14). Unpaired t test with Welch's correction performed. * denotes statistical significance. Statistical significance defined as p=<0.05

MAP Young vs old		Diameter Young vs Old	
Measure	**P value**	**Measure**	**P Value**
Baseline [1]	0.25	Baseline[1]	0.05
AVG MAP [2]	0.37	AVG Diameter$_2$	0.04*
Max MAP [3]	0.19	Max Diameter[3]	0.03*
Min MAP	0.65	Min Diameter	0.76

[1]Baseline defined as baseline period before CPT

[2]Average defined as average increase throughout the CPT

[3]Max defined as the maximum increase throughout the CPT.

AVGMAP% vs Cardiovascular Disease Risk Variables

To investigate the relationship between MAP and CVD risk factors, linear regression analysis was performed. This investigated the relationship between average increase in MAP and a number of factors known to be associated with cardiovascular risk. The MAP expressed as an average of the increase in MAP above baseline and was expressed as a percentage (AVGMAP%) There was no correlation between AVGMAP% and any of the cardiovascular disease associated risk factors in this group. **(*Table* 6)**

***Table* 6)** Pearson linear relationship between the average increase in MAP throughout the CPT when compared to baseline MAP expressed as a percentage (AVGMAP%) compared to cardiovascular risk factors.

AVGMAP% vs Variable	R2 Value	P Value
AVGMAP% vs Systolic BP	0.01	0.63
AVGMAP% vs BMI	0.02	0.81
AVGMAP% vs CAR%	0.08	0.08
AVGMAP% vs MAP	0.01	0.78
AVGMAP% vs QRISK Score	0.02	0.66

Association of Blood Pressure and Cardiovascular Risk

"High" vs "Low" Blood Pressure and Cardiovascular Risk Factors

To investigate the relationship between change in MAP throughout the CPT. The participants were divided according to the relative increase in blood pressure in response to the CPT. In this study, "Low relative" BP response was defined as <15% increase (n=8), "Medium relative" BP response defined as a 15% and 30% change (n=20) and a "high relative" response defined as greater than 30% (n=12). All responses were percentage increase in (MAP) above baseline MAP throughout the CPT. There was a statistically significant difference in baseline MAP (P = 0.053), Peak MAP (P = <0.001) and Relative ΔMAP (P = <0.001) in the low relative, medium relative and high relative *Δ*BP groups.

***Table* 7)** CAR% and blood pressure results of the groups divided based on relative ΔMAP (low= <15%, medium = between 15% and 30%, high = >30%). Relative ΔMAP was defined as the percent increase from baseline to the peak MAP. Unpaired T Test performed. **A**) Post-hoc significantly different from group 1 **B)** Post-hoc significantly different from group two.

	LOW RELATIVE ΔBP (N=8)	MEDIUM RELATIVE ΔBP (N=20)	HIGH RELATIVE ΔBP (N=12)	P-VALUE
Age (years)	42.61 ± 9.31	40.92 ± 9.21	44.33 ± 10.61	0.61
Weight (kg)	83.51 ± 12.32	81.21 ± 11.73	83.92 ± 15.32	0.82
Height (meter)	1.76 ± 0.08	1.77 ± 0.07	1.79 ± 0.07	0.60
BMI (kg/m^2)	26.71 ± 2.21	26.01 ± 3.54	26.12 ± 4.65	0.89
Positive family history (yes)	1.51± 0.76	1.23 ± 0.83	1.34 ± 0.81	0.66
Baseline diameter (cm)	0.67 ± 0.05	0.65 ± 0.05	0.69 ± 0.08	0.22
CAR (%)	1.33 ± 4.63	4.51 ± 4.51	6.42 ± 6.53	0.11
Baseline MAP (mmHg)	95 ± 11	85 ± 8	90 ± 9	0.05*
Peak MAP (mmHg)	102 ± 13	106 ± 10	125 ± 15[ab]	<0.01 *
Relative ΔMAP (%)	7.54 ± 4.32	23.71 ± 3.92[a]	38.01 ± 4.00[ab]	<0.01 *

***Table* 8)** Mean plus/minus SD MAP and Diameter data of participants when split into groups according to their ΔMAP (low= <15%, medium = between 15% and 30%, high = >30%). Relative ΔMAP was defined as the percent increase from baseline to the peak MAP. One-way ANOVA performed.

	Low relative ΔBP (n=8)			Medium relative ΔBP (n=20)			High relative ΔBP (n=12)		
	Baseline	**AVGCPT**	**MaxCPT**	**Baseline**	**AVGCPT**	**MaxCPT**	**Baseline**	**AVGCPT**	**MaxCPT**
MAP (mmHg)	94±11	94±13	102±13	85±8	97±9	106±10	90±9	113±14	125±15
Diam (cm)	0.69±0.05	0.68±0.06	0.69±0.07	0.65±0.50	0.66±0.05	0.68±0.05	0.69±0.08	0.71±0.07	0.73±0.06

***Table* 9)** Statistical comparison investigating overall effect of MAP and Diameter during baseline, AvgCPT and MaxCPT in participants grouped "low", "medium" and "high" according to their ΔMAP. One-way ANOVA performed.

MAP Low, Medium, High		Diameter Low, Medium, High	
Measure	**P Value**	**Measure**	**P Value**
Baseline	0.05	Baseline	0.22
AVGCPT	0.01*	AVGCPT	0.08
MaxCPT	<0.01*	MaxCPT	0.06

Differences in Characteristics in Whole Group, "Dilator" and "Constrictors"

The participants were then separated using their Carotid Artery Response (CAR%) to the CPT. The "dilator" group were defined as participants who demonstrate a positive CAR% to the CPT (N= 34). The "constrictor" group were participants who demonstrate a negative CAR% to the CPT (N=6). Independent Samples T Test performed. There was no significant difference in any of the demographic measures in these two groups (***Table 10***). There was a statistically significant difference in CAR% in these two groups (5.7± 4.7 in "Dilators" and -2.8± 2.48 in the "Constrictor" group. P = <0.001) (***Table 10***).

***Table* 10)** Participant characteristics and CAR% when divided into groups based on the presence of diameter dilation or vasoconstriction. P-values refer to an unpaired t-test comparing Dilator vs Constrictor groups.

	Total (n=40)	Dilator (n=34)	Constrictor (6)	P-value
Age (years)	42.25±9.53	42.08±9.31	43.17±12.01	0.90
Weight (kg)	82.46±12.73	81.05±11.24	90.32±18.71	0.34
Height (meter)	1.77±0.07	1.78±0.07	1.75±0.05	0.65
BMI (kg/m^2)	26.16±3.63	25.58±2.91	29.37±5.32	0.06
Number of risk factors	0.43±0.30	0.44±0.50	0.17±0.41	0.37
Baseline diameter (cm)	066±0.06	0.67±0.05	0.66±0.10	0.43
CAR (%)	4.42±5.42	5.71±4.73	-2.92±2.48	<0.01*
Baseline MAP (mmHg)	84.53±22.23	88.72±7.43	91.51±82.12	0.46
Peak MAP (mmHg)	111±15	110±15	109±19	0.29
Relative ΔMAP (%)	14.12±14.21	22.01±10.43	20.23±15.21	0.518

***Table* 11**) Correlation between CAR% and the blood pressure variables during CPT for all participants, constrictor and the dilation group. ΔBP is the absolute difference between peak and baseline blood pressue, whereas relative ΔBP is the percent increase from baseline to the peak blood pressure. Pearson correlation.

	All participants (n=40)		Dilation group (n=34)		Constrictor Group (n=6)	
	Pearson correlation	**p-value**	**Pearson correlation**	**p-value**	**Pearson correlation**	**p-value**
Baseline diameter	-0.35	0.03	-0.59	<0.01*	0.16	0.76
Baseline MAP	0.21	0.20	0.30	0.09	0.22	0.67
Peak MAP	0.36	0.02*	0.42	0.01*	0.46	0.36
ΔMAP	0.33	0.04*	0.33	0.06	0.43	0.39
Relative ΔBP	0.27	0.09	0.23	0.20	0.65	0.16
MAP change$_A$	0.33	0.04*	0.27	0.12	0.45	0.35

5.10. Discussion

The primary aim of this study was to investigate the relationship between changes in blood pressure and carotid artery diameter during the CPT. This study presented the following key findings. First, the start of dilation and the timing of the peak carotid artery diameter response preceded blood pressure changes during the cold pressor test. Second, there were no differences in baseline characteristics including age, weight, BMI or in the magnitude of carotid artery dilation when comparing groups based on the magnitude of blood pressure increase. This finding was supported by the lack of correlation between the relative changes in carotid artery diameter and blood pressure during the CPT. Finally, individuals who demonstrated carotid artery vasoconstriction demonstrated a comparable increase in blood pressure to those who dilate during sympathetic stimulation. Taken together, this study suggested that the characteristic increase in blood pressure during sympathetic stimulation did not significantly contribute to carotid artery vasomotion in healthy men aged 31-59. However, it should be acknowledged that there was no significant difference in risk factors or BMI when groups were separated in to "dilator" vs "constrictor" groups or when participants were grouped according to their blood pressure response. There was also no significant difference in QRIsk3 score (A score used clinically to predict CVD risk in asymptomatic individuals) in the "dilator" vs "constrictor" group ($p=0.4096$) (Data not shown). It also should be noted that confounding variables not measured such as exercise and diet may also have an impact on the lack of correlation between BP and CAR% (150)

The CPT is a frequently used procedure to activate the SNS in humans. As expected, and in line with several previous studies, blood pressure gradually increased after a period of 20-30 seconds. The increase in blood pressure was most likely the result of (nor)adrenaline release, mediating a vasoconstriction response in peripheral arteries that cause an increase in total peripheral resistance (151, 152). Interestingly, the timing of the start of carotid artery dilation, and the timing of the peak diameter change, significantly preceded the blood pressure response. This suggested that contrary to our hypothesis, carotid artery response was not directly linked or driven by the increase in blood pressure response during the cold pressor test. To further support this conclusion, there was no relation between the degree of blood pressure increase and the CAR% during the CPT. A final strong argument against a key role for blood pressure in mediating the carotid artery vasomotor response during the cold pressor test was the presence of vasoconstriction in some individuals. A significant increase in blood pressure was found in these individuals, which did not differ from the blood pressure response found in subjects with carotid artery dilation. This study involved healthy participants with no previous history of cardiovascular disease. However, it should be noted that the average BMI of these participants is 27±4. This means that on average, the participants were overweight. This may have an impact on endothelial function and therefore may be a confounding variable.

Despite the absence of a relation between the diameter and blood pressure response, both responses seemed strongly related to sympathetic stimulation. Indeed, catecholamine-release during sympathetic stimulation seem directly related to carotid artery responses (153). This catecholamine release may also be responsible for the increased peripheral artery resistance and blood pressure changes (153). Differences in sensitivity of receptors or mechanisms contributing

to vasomotion between central (i.e. carotid) and peripheral arteries may explain the difference in timing of the blood pressure and diameter responses. Nonetheless, given their dependence on catecholamines (154), it was expected a relationship between the magnitude of blood pressure and diameter response would be identified. One potential explanation for the lack of relation is that catecholamine-release was less strongly related to vascular responses than anticipated. Cummings et al. (1983) found adrenalectomised participants do not demonstrate an increase in adrenaline, noradrenaline or dopamine during the CPT, despite the presence of an increase in blood pressure of comparable magnitude to healthy individuals. Therefore, peripheral artery responses (and therefore blood pressure) to the CPT may be independent of catecholamine release (155), whilst catecholamines seem essential for carotid artery diameter responses. At least, this study suggested no direct link between blood pressure and carotid artery diameter response to the CPT in healthy individuals.

An important factor to consider is that structural properties of the artery may influence the dilator response. A previous study demonstrated a negative correlation between baseline carotid diameter and CAR% in non-diseased average risk, high risk, and coronary artery disease patients, but not with the carotid artery intima-media wall thickness (IMT) (109). In contrast, Van Mil et al. reported no correlation between the baseline carotid diameter or IMT and CAR% in healthy people (115), whilst others also found no correlation between coronary artery baseline diameter and dilation response (143). In this study, there was a significant, but weak, inverse correlation between the baseline carotid diameter and the CAR% in the whole group, implying that a smaller baseline diameter correlates with a larger CAR%. This observation fits with several previous studies examining peripheral arteries, where a smaller brachial or femoral artery correlates to a

larger flow-mediated dilation (156, 157). The presence of a correlation between diameter and CAR% in this study, whilst largely absent in previous work, may relate to the inclusion of healthy individuals only. For example, previous work in peripheral arteries also found a weaker or non-existing correlation between baseline diameter and dilator responses in older and diseased populations (158). This may be explained by the impact of older age and/or cardiovascular risk factors in those groups. This may affect both baseline diameter and dilator response. Consequently, affecting the (weak) inverse relation between both parameters in healthy young individuals. At least, this study suggests that structural characteristics of the artery wall should be considered when examining the CAR% responses, but unlikely affect or interfere with the blood pressure increase (and subsequent diameter response) during the CPT. Due to scan quality, structural analysis such as IMT was not possible.

Limitations.

One potential limitation in this study was that the results of our study only apply to healthy men, making extrapolation to other groups as well as women difficult. This is important because distinct populations have demonstrated different CAR% and/or blood pressure response (159, 160), whilst also physical activity may affect the blood pressure and/or CAR% (161). Nonetheless, it seems unlikely that these confounding factors, despite their role in changing blood pressure and/or CAR%, affected the relationship between the blood pressure and diameter increase during the CPT. Another limitation was that our study did not explore the causal link between blood pressure and CAR%. To investigate this, a study would require direct manipulation of the blood pressure response during sympathetic stimulation. Monahan et al (2013) examined the

impact of the CPT on the left anterior descending artery with α- and β-adrenergic antagonists (139) and found adrenergic blockade to abolished coronary artery vasodilation. In agreement, Van Mil et al (2018) examined carotid and coronary artery responses during the CPT with and without α_1-receptor blockade, and reported abolished carotid and coronary artery responses when combined with α_1-receptor blockade (113). Whilst this provided evidence for the role of adrenergic receptors in contributing to carotid (and coronary) artery vasodilation during the cold pressor test, the presence of an increase in blood pressure during blockade hampered conclusions pertaining to the role of blood pressure per se.

In conclusion, findings from this small pilot study suggested that carotid artery diameter changes during the CPT are unlikely related to the characteristic increase in blood pressure. The start and peak of the diameter preceded that of the blood pressure, whilst no correlation was present between the magnitude of the blood pressure response and CAR%. Moreover, even individuals who presented carotid artery vasoconstriction demonstrated an increase in blood pressure, making it unlikely that the blood pressure rise should be regarded as the dilator stimulus. This study added evidence that CAR% provides a measure of vasomotion, independent of the increase in blood pressure during the CPT.

6. Chapter 6: Correlation of Carotid Artery Response to Surgical Complexity and Outcomes in Patients undergoing Coronary Artery Bypass Graft Surgery

6.1. Chapter introduction:

This chapter describes a clinical study investigating if CAR% predicts risk in patients undergoing CABG surgery.

6.2. Research question:

Does pre-surgery assessment of the CAR% predict post-surgery outcomes (i.e. 30-day AEs) in patients with coronary artery disease undergoing CABG surgery?

6.3. Aims:

To explore the prognostic value of carotid artery reactivity (CAR%) to predict post-surgical AEs in patients with CAD undergoing CABG surgery combined with valve surgery.

6.4. Objectives:

1) **Does a reduced CAR% result in a higher incidence of AEs in patients undergoing CABG combined with valve surgery?**

   To explore if a reduced CAR% is associated with a higher incidence of AEs in patients undergoing CABG combined wtih valve surgery

2) **Does CAR% correlate with EuroSCORE II score in patients undergoing CABG combined with valve surgery?**

To explore if CAR% predicts AEs more accurately than EuroSCORE II in patients undergoing CABG.

6.5. Hypothesis

H_1 CAR% Predicts short term (30 day) incidence of AEs post CABG-surgery

H_2 CAR% better predicts risk than EuroSCORE II, a risk stratification tool currently utilized clinically.

6.6. Abstract

Coronary artery endothelial function has been demonstrated to predict risk of disease progression and survival in a variety of clinical groups (109). In health, there is an endothelium-dependent dilation. Whereas, in patients with cardiovascular disease risk, this is reduced. In patients with severe coronary artery disease, there is a paradoxical constriction (14, 109). This is measured invasively using quantitative angiography. The carotid arteries may be uniquely placed to offer a simple imaging target with both structural and functional similarities to the coronary arteries (14). The carotid and coronary arteries also demonstrate a similar response to SNS stimulation (112). Recently, our group demonstrated that CAR% predicts risk of disease progression and AEs in patients with PAD (54). The aim of this study was to investigate if CAR% predicts risk in patients with central (coronary) artery disease undergoing coronary artery bypass graft surgery (CABG). A sample of 95 participants were included in analysis. Participants underwent provocation testing using the CPT alongside CAR%. Patients were then followed-up for incidence of AEs and survival for 30 days post-operatively. There was a weak inverse correlation between CAR% and EuroSCORE II (P: 0.01 r: -0.26). EuroSCORE II (current clinical practice) did not predict AEs in this group (ROC_{AUC}: 0.60). CAR% also did not predict risk in this group (ROC_{AUC}:0.51). When combined, CAR% marginally improved predictive ability (ROC_{AUC}:0.615). Although this was still not predictive of events. This may have been due to the small number of events within our study (ten total). It may also have been that CAR% alone does not predict AEs but when combined with current risk prediction tools (EuroSCORE II) this improves the predictive capacity. This requires further work.

6.7. Background

Coronary Artery Bypass Graft Surgery

Coronary Artery Bypass Graft (CABG) Surgery is one of the most common cardiac surgical interventions undertaken with approximately 800,000 operations undertaken world-wide each year (162). CABG surgery is offered on the grounds of symptom relief and prognostic benefits in patients where medical therapy or percutaneous coronary intervention (PCI) are unsuccessful or inappropriate (163). In CABG surgery, an artery or vein is used from elsewhere within the body to bypass the flow-limiting lesion in the coronary artery. This procedure restores flow to the distal tissue providing both symptom relief and prognostic benefit (9). The process of coronary artery bypass graft surgery and conduit vessel selection are discussed extensively elsewhere (9). A second common dysfunction within the heart is valvular regurgitation/stenosis. The cardiac valves are responsible for controlling blood flow around the heart and are instrumental in avoiding retrograde flow within the heart. However, these valves can become dysfunctional and allow regurgitation and subsequent heart failure. Surgical interventions to repair or replace these valves aim to restore function thereby restoring directional flow throughout the heart. CABG surgery is the most commonly undertaken cardiac surgical intervention whereas CABG combined with valve surgery is the most common combined cardiac surgical procedure (164). There is a significant body of research investigating the characteristics of CABG surgery. This is key as CABG is offered to a wide variety of patients with a large variability in their risk profile. It is also key that patient characteristics including risk profile are reported in prognostic studies to allow individual prognostic studies be understood in the context of the larger literature.

This is in-line with the PROGnosis Research Strategy (PROGRESS) framework for undertaking and reporting prognostic studies (165, 166). It is therefore key that risk prediction tools are understood in the context of the risk profile of individual research studies and are not over-extrapolated. One of the largest meta-analyses investigating characteristics of patients undergoing CABG surgery have been used to characterize approximately 32000 patients undergoing CABG surgery (167).

Risk and Risk Prediction in CABG Surgery

Although CABG surgery is a commonly utilised treatment for CAD, it is associated with some risk. According to a large meta-analysis of 13327 procedures in North American, cumulative short-term (30 day) risk of operative mortality in isolated CABG surgery is approximately 2.1% in men and 3.9% in women (167). Whereas short-term (30 day) cumulative risk of AEs, are 2.4% risk of MI, 1.3% risk of stroke, 1.5% risk of GI bleed and 0.8% risk of renal failure (162). This 2-4% risk for AEs within the first month post-CABG is relatively high, especially considering the good long-term survival of these patients with a 5 year-, 15 year-, 25 year- and 35 year survival of 86% , 48% 19% and 7% respectively (10).

There are a number of risk stratification tools available to risk stratify patients undergoing CABG surgery. These risk stratification tools are typically derived from large epidemiological datasets that indicate factors that affect clinical outcomes. These risk stratification tools use measures that can be broadly categorised into: 1. clinical status (including past medical history), 2. modifiable risk factors, and 3. non-modifiable risk factors. Clinical status includes past medical history, disease severity and operative urgency alongside symptom/disease severity. This includes the Canadian Cardiovascular Society (CCS) score of angina severity and the New York

Heart Association (NYHA) dyspnoea score (168, 169). The modifiable factors include smoking status and BMI (169). Non-modifiable risk factors include age and sex and past medical history (169). All of which negatively affect general, cardiac and vascular health. However, it remains a challenge to develop a valid risk stratification tool that performs accurately across the spectrum of low-, intermediate-, and high-risk patients (45, 170-174). The two most commonly utilized tools in clinical practice are the Society for Thoracic Surgeons (STS) score (typically used within the United States of America) and the EuroSCORE II risk calculator (most often used within Europe) (35). EuroSCORE II focuses mostly on non-modifiable risk factors including age, past medical history and clinical status and does not differentiate between cardiac surgical procedures/techniques. The STS scoring system is a more in-depth system which includes non-modifiable risk factors, past medical history and clinical status. The STS scoring system also allows clinicians to differentiate between surgical procedures (169).

The ability of both the EuroSCORE II and STS to predict risk has been extensively investigated. A large meta-analysis comparing EuroSCORE II and STS, comparing the predictive ability of 22 studies was undertaken. They found that EuroSCORE II and STS scored comparatively (with a summary difference in AUC of 0.00) and were both able to predict risk in patients undergoing CABG surgery (169). This meta-analysis also found EuroSCORE II outperformed STS in respect to calibration (O:E ratio 0.94 and 0.75 respectively), whereas the STS outperformed EuroSCORE II in discrimination (AUC: 0.75 and 0.73 respectively) (169). Epidemiological-derived datasets have been demonstrated to be unable to predict risk of sudden cardiac death (174). Importantly, it has also been demonstrated that 22% of 215 participants investigated with coronary Computed Tomography (CT) were demonstrated to have significant coronary artery atherosclerosis (174).

Of these, thirteen (25%) and 30 (58%) with significant stenosis were classified into National Cholesterol Education Program as low risk and mild coronary calcification respectively (174). This highlights the under-performance of epidemiology-derived datasets. Epidemiological-derived datasets may also be unable to predict risk in low and moderate risk patients (170-175). Taken together, this highlights the imprecision in risk stratification using epidemiological models. Similarly, risk stratification tools rarely measure endothelial-protective factors such as exercise and diet (14, 15). This highlights the need to investigate the vascular health of individual patients thereby allowing a more personalized and accurate risk score. This may be achieved through measuring endothelial function as a surrogate of vascular health in these groups. It has previously been demonstrated that endothelial function has a profound effect on outcomes and survival in healthy individuals, those with cardiovascular disease (CVD) and those undergoing cardiac surgery (42, 109, 176).

Endothelial Function as a Risk Predictor

The importance of measuring endothelial function as a method to predict risk of AEs was first established using invasive quantitative angiography. This was performed first in combination with the endothelium-dependent vasodilator acetylcholine (ACh). Schachinger et al (2000) demonstrated patients who demonstrate coronary artery constriction in response to ACh infusion or cold pressor test had a significantly increased risk of incidence of cardiovascular disease ($p=0.09$ and $p=<0.01$ respectively). Events defined as cardiovascular death, acute coronary syndrome, MI, PCI or CABG, ischaemic stroke or peripheral revascularization. These patients also had a blunted response to both increased blood flow ($p=<0.01$) and intracoronary

infusion of nitroglycerin (p= 0.01) (42). This work was further supported by Suwaidi et al (2000) who found that severe endothelial dysfunction in the absence of obstructive coronary artery disease is associated with an increased risk of cardiac events (65). Events defined as MI, percutaneous or surgical revascularization or cardiovascular death. Quantitative angiography in response to pharmacological (ACh) or physiological (cold pressor test) stimulation became the gold standard in measuring coronary artery endothelial function (42, 65). Schindler et al (2005) investigated myocardial blood flow (MBF) throughout the CPT using Positron Emission Tomography (PET) (52). Adverse events were defined as cardiovascular death, acute coronary syndrome, MI, PCI, CABG, ischaemic stroke or peripheral revascularistion. They found that, in patients with normal coronary angiograms, impaired increase in MFB is associated with an increased risk of developing cardiovascular events in patients undergoing diagnostic cardiac catheterization (52)

Table 12) Summary table of studies investigating the predictive capacity of endothelial function to predict risk.

Study	Population	Measure	Outcomes	Follow up	Results
Schachinger (42)	CAD[5] patients	Quantitative angiography, CPT[1] and ACh[2]	CVD death, ACS[4], MI, CABG, stroke, peripheral revascularisation	7.7 years	Significant difference in AE vs non-AE Groups ACh: P: 0.009 CPT: P: <0.001
Suwaidi (65)	Mild CAD[5]	Quantitative angiography and CPT[1]	MI, PCI, surgical revascularization	2 years	Significant difference in AE vs non-AE groups P: <0.05
Schindler (52)	Mild CAD[5]	Myocardial blood flow to CPT[1] measured with PET[3]	CVD deaths, MI, PCI, CABG, stroke, peripheral revascularasiation	5.5 years	Significant difference in AE vs non-AE groups P:0.033 log-rank

[1] Cold Pressor Test, [2] Acetylcholine, [3]Positron emission tomography, [4] Acute coronary syndrome, [5] Coronary artery disease

Clinical utility of risk prediction and issues with inaccurate risk prediction

Accurate risk prediction is key to all areas of healthcare from individual clinician and patient decision making to service planning and evaluation (177, 178). A risk prediction tool has to be sensitive, specific and reproducible (179, 180). The sensitivity of a test is the ability of a test to identify true positives within a dataset. In the context of surgical risk prediction, this is the number of participants that are accurately identified as being “high risk”. These are also referred to as “true positives”. The specificity of a test is the ability to identify true negatives within a

dataset. In the context of surgical risk prediction, this is the ability to correctly identify patients that are low risk.

The implications of risk prediction can be categorised in to three categories. One, informed consent, two the level of care and three four service planning.

Informed consent

Accurate risk prediction is key in informed consent. First, a core tenant of clinical decision making is the quality and accuracy of the data used to make that decision. This is a collaborative process between clinicians, patients and their friends or relatives. This is reliant upon both parties making a decision based on accurate and reliable information (179). This is a fundamental tenant of informed consent. If risk prediction tools are inaccurate, this means decision making is fundamentally flawed (180). Second, a key part of the informed consent process is ensuring that language and concepts are understood by everyone involved including the patients and their families. The majority of evidence-based practice is reliant upon complex statistical analysis which is often difficult to communicate to laypersons. However describing adverse events as a frequency of total interventions undertaken may be easier to communicate (180).

Levels of care

A second key consequence of risk prediction is offering appropriate evidence-based practice and level of care/intervention for each individual and allowing a personalised patient-centered approach to risk and care for each individual. The ability to accurately identify patients that are at an increased risk is important in all areas of decision making. The ability to identify high-risk patients means that a more appropriate treatment can be undertaken. This may mean offering

more aggressive interventions thereby reducing risk. If these patients are not identified, this may mean "under treating" these patients. This may mean that they experience more events (181). Similarly if a patient is considered to be unacceptably high risk for surgical intervention, this may mean that they can undergo a more conservative method of treatment (181). If patients are investigated and found to be very low-risk, this may mean that they can undergo a more conservative management thereby avoiding the inherent risks associated with surgical intervention (181). This avoids the risk of "over-treating" patients who are sufficiently low risk to undergo more conservative management. This may mean that patients who are at an increased risk may be identified and may further identify key targets for optimisation such as cardiovascular fitness, smoking cessation or optimal medical management (182-186) .

Service planning

As discussed above, risk prediction tools are key in decision making for both individuals and clinicians, however risk prediction tools also play a key role in service planning and performance analysis. The identification of the highest risk individuals may allow for extensive pre-planning such as further tests and patient optimisation and ensuring that the highest-risk patients are cared for by highly-specialist. This may require a combined approach through multiple specialists or through care in a specialist tertiary referral center (181). The identification of higher risk patients can also allow the safe and effective management of patients including the pre-planning of those that are likely to require extended Intensive Care Unit stay (180).

Service auditing

The utilisation and publication of outcome data for individual surgeons and surgical units allows for external auditing. This allows the comparison with other individuals and institutions to ensure

that outcomes fall within defined parameters. If individuals or institutions fall outside of acceptale parameter, it may identify a need for further training or service planning (181).

Endothelial Function and Surgical Risk

Endothelial function has been demonstrated to predict risk of AEs in both non-diseased and a variety of diseased groups (42, 45, 187). There are a small number of studies that have investigated endothelial function as a method to predict risk in surgical patients. Saito et al (2018) investigated Reactive Hyperemia Peripheral Arterial Tonometry (RH-PAT) in 197 patients who underwent cardiac surgery (188). It has previously been demonstrated that an RHI <1.67 was an indicator of endothelial dysfunction (188). In this group, RHI ≤1.64 had significantly more AEs than those with an RHI >1.64. Multiple regression analysis also demonstrated that the number of surgical procedures together with an RHI ≤1.64 as significant predictors of AEs (188). Similarly, McIlroy et al (2013) investigated RH-PAT in 238 subjects undergoing intermediate to high-risk non-cardiac surgery. They found endothelial dysfunction investigated via RH-PAT provided a limited discrimination for identifying subjects with myocardial injury after non-cardiac surgery (MINS) (ROC_{AUC} 0.60, 95% CI – 0.47-0.73) (189). An RHI of ≤1.22 provided a sensitivity of 31% and specificity of 96% with a positive diagnostic likelihood of 8.0. Endothelial function was also a potent predictor of MINS (OR 11.2, 95% CI: 4.1-30.5, p=0.001) (189). They also found that RH-PAT combined with Lee index (a prospective validated model to predict cardiac risk in surgical patients) improved predictive capacity over Lee Index alone (c statistic 0.69 vs 0.77 respectively. Integrated discrimination improvement 0.11 p=0.003) (189). However, the same group also investigated RH-PAT in 218 participants undergoing intermediate or high-risk non-cardiac

surgery. They investigated renal dysfunction/failure as a primary endpoint and found RH-PAT did not predict risk of renal dysfunction/failure in this patient group (190). There are also a small number of studies that have investigated the ability of Flow Mediated Dilation to predict risk in a variety of surgical patients. Firstly, Gokce et al (2002) investigated endothelial function via Flow Mediated Dilation (FMD) in 187 patients undergoing vascular surgery. They followed-up patients for 30 days post-operatively for incidence of AEs and survival. They found FMD was significantly lower in patients who had AE(s) in the 30-day post-operative period (4.9±3.1 vs 7.3±5% respectively: p=<0.001) whereas endothelium-independent vasodilation to nitroglycerin was not significantly different in this group (191). Suggesting a normal functioning VSMC in the context of a dysfunctional endothelium. FMD was also shown to independently predict risk of Aes in this group (p=0.01). When FMD cut-off was defined as 8.1%, FMD had a 95% sensitivity, 37% specificity and a 98% negative predictive value (191). Similarly, Schier et al (2012) investigated Brachial Artery Reactivity Testing (BART) in 63 patients undergoing major thoracic surgery. Patients who demonstrated a "low" FMD (<11.5%) had a significantly higher incidence of post-operative complications (54% vs 30% p=<0.001) (192). Taken together, this may suggest that non-invasive measures of endothelial function may be predictive of Aes and survival in patients undergoing surgical procedures. This highlights the ability of non-invasive measures of endothelial function to predict risk in both cardiac and non-cardiac surgery

Carotid Artery Reactivity Testing

As discussed above, although a number of measures have shown some potential prognostic benefit, these are broadly are prohibitively expensive or investigated non-invasively using

peripheral arteries (14, 42). A significant limitation in using peripheral arteries as a surrogate of coronary artery endothelial function is the dissimilarity in both structure and function in peripheral vs central arteries. This is reviewed extensively within the "*Background*" section of the PhD and in a review article written as part of this PhD (14). The carotid arteries may be uniquely placed to offer an easily accessible central artery with both structural and functional similarities to the coronary arteries. This was first investigated by Rubenfire et al (2000) (109) who found that, in participants with no history of cardiovascular disease, these participants demonstrated a carotid artery dilation in response to the SNS stimulus. Whereas, in high-risk participants the carotid artery response was attenuated and in those with significant coronary artery disease, participants demonstrate a paradoxical constriction of the carotid arteries similar to that in the coronary arteries (109). To further investigate this, Van Mil et al (2018) investigated the relationship between carotid artery diameter measured using vascular ultrasound and left anterior descending artery blood velocity measured using transthoracic doppler in 33 healthy individuals. They found a moderate correlation between carotid artery diameter and LAD artery blood velocity (r=0.486, and 0.402 respectively) (112). This led them to then investigate carotid artery reactivity as a method of predicting risk in patients with PAD. They found that the "constrictor" group had a 4.1-fold increased risk in CV events and a 2.0-fold increased risk in disease progression when compared to the "dilation" group (54). This was the first study to investigate CAR% as a method to predict risk in clinical populations.

Both Rubenfire et al (2000) and Van Mil et al (2018) have demonstrated that carotid artery response can predict risk of Aes in patients with cardiovascular disease and those with PAD. This therefore led to us to use CAR% as a method to investigate endothelial function in patients with

central (coronary) artery disease undergoing isolated CABG or combined CABG combined with valve surgery. Our hypothesis was that first, CAR% predicts the short-term (30 day) incidence of AE's in patient's post-CABG surgery and second, that CAR% better predicted risk than the European EuroSCORE II risk calculation tool.

6.8. Methods

Progress Framework

This research project was reported within the PROGnosis RESearch Stratergy (PROGRESS) framework (193). This is a conceptual framework developed collaboratively by experts in prognostic research. This is developed to outline the importance of prognostic studies and to provide a framework for the development of prognostic studies, requirements when designing and undertaking prognostic research studies and the multiple phases employed in prognostic research. This is outlined in PROGRESS Framework 2 – Prognostic Factors. This describes the process from model development, model validation and finally investigating the impact of the prognostic model on research and clinical outcomes (165). When undertaking prognostic research, it is key that factors are investigated in participants with a given start point, in this case patients with CABG plus or minus valve surgery. This has to have specific, clinically relevant endpoints. The endpoints are discussed within the "Methods" section of this chapter. It is also key to clearly define and consider the limitations of this study as well as prognostic research more broadly including publication bias, reporting bias and adequate statistical analysis.

A key consideration in the PROGRESS framework is prognostic research as a process, rather than as a single study. This therefore describes PROGRESS research in three phases (166). First, in

model development, second in the internal and external validation of the tool and finally by investigating the impact of this tool in clinical practice. The PROGRESS framework also requires the development using a large dataset, based on a study protocol with sound statistical planning and finally to be validated in independent datasets obtained from different locations. This study can be considered as an early first step or pilot study. As discussed throughout this thesis, there are key future steps required to fully investigate the predictive capacity of this test.

Study Design

A longitudinal observational study design was employed. All participants are identified, recruited and undergo testing prior to surgery. All participants are then followed-up post-operatively for a 30-day period to investigate if CAR% predicts risk in CABG patients.

Ethical Approval

Ethical approval was sought and gained through NHS ethics committee, and governance approval from Health Research committee. Reviewed by the North West – Liverpool Central Research Ethics Committee on 28/6/17 (17/NW/0347).

Public and Participant Engagement

The impact of public and patient involvement (PPI) in the development of clinical trials has been demonstrated to improve enrolment, quality, relevance and uptake of research (194). The study was therefore developed in collaboration with clinicians, managers and the Service Users Research Endeavour (SURE) group. The SURE group are a group of patients and their family members responsible for patient advocacy in research throughout Merseyside. The project was

also developed in collaboration with service managers at LHCH to maximize recruitment whilst minimizing impact to clinical services.

6.9. Study Setting

Recruitment was undertaken at the Liverpool Heart and Chest Hospital NHS Foundation Trust (LHCH). All participants were identified as requiring Coronary Artery Bypass Graft (CABG) surgery or CABG combined with Valve surgery by clinicians at LHCH.

Participants

Eligibility

We excluded participants who had CABG surgery combined with other forms of major surgery such as aortic root surgery as this would skew the risk of AE's of during surgery and in the post-operative course. We excluded patients who had undergone carotid artery endarterectomy, carotid artery surgery or with known or suspected Raynaud's syndrome. We also excluded those below the age of 18 or those who cannot give consent. This is in line with previously published research (43, 54, 130).

Participant Identification

Participants were recruited through both the outpatient and inpatient departments at LHCH. Participants were recruited initially through the outpatient department at LHCH. Participants received a participant information sheet at least 24 hours prior to testing. The use of outpatient clinics limited recruitment due to practical considerations and availability. Therefore, recruitment was subsequently expanded to also include inpatients at LHCH. Paper consent forms were kept securely at Liverpool John Moores University.

Variables

Participant Preparation

The research protocol was undertaken in a temperature and light-controlled room. All participants were asked to lay supine for at least five minutes prior to testing as per previously published research and guidelines (49, 54, 130). Participants were also asked to abstain from food or drink known to affect endothelial/SNS function, although they were allowed to eat. This was in-line with guidelines for investigating endothelial function and previously published research (54, 130). Although medication was not altered. The medication was not recorded as this is recorded on a separate tab within the electronic patient system and access was not given.

Data Sources

The past medical history, angina status (Canadian Cardiovascular Society score) and dyspnoea status (NYHA classification) were recorded for each participant prior to testing. This was in-line with National Institute for Cardiovascular Research (NICOR), National Adult Cardiac Surgery Audit (NACSA) dataset. This is detailed in the "Participant Follow Up" section below.

Carotid Artery Ultrasound alongside the Cold Pressor Test was undertaken as per the *Methods* section of this thesis. The carotid artery diameter alongside RBC velocity were measured non-invasively using the left common carotid artery imaged via ultrasound. Participants underwent the cold pressor test including a one minute "baseline" recording, two minutes with the left hand submersed in water at 4°C for two minutes. Due to practical considerations, the submersion time for the hand during the CPT was reduced to two, rather than three minutes. This maintained patient safety whilst also ensuring sufficient SNS stimulation (54). In our study investigating CAR%

in healthy participants, alongside other studies have all shown that there is a statistically significant change in both blood pressure and CAR%. In our first study investigating CAR%, peak MAP occurred at 112±38 seconds. The peak CAR% occurred at 92±43 seconds. Therefore, an immersion time of 2 minutes was considered sufficient to achieve peak CAR%.

Data quality

Scan acquisition

An area distal to the carotid bulb is used for scan acquisition. An area adjacent to, but not including the carotid bulb is used. The largest region of interest possible is used. This is at least one third of the entire segment visualised. This ensured that data is representative of the entire arterial segment.

Scan analysis

All scans were analysed blinded to avoid bias in analysis. All scans were also analysed independently by two researchers to ensure quality and further reduce the probability of bias. As all scans were only undertaken once, it was not possible to measure between-scan variation.

Clinical data collection

Patient data was collected from electronic patient records systems. The use of electronic patient records alongside specific post-operative protocols ensured that data was up to date and accurate. There were no “missing” datapoints. A random selection of 30 participants were selected and the data input and follow-up was checked independently by a second researcher using the patient medical records. This ensured accuracy and validity in data collection.

Participant Follow Up

To enable participant follow-up, all participants included within the trial were added to an online database on the Electronic Patient Records (EPR) system at LHCH. This allowed the accurate follow-up of each participant involved in the trial. All participants were followed-up using the secure online EPR system. The pre-operative risk factors, intra-operative surgical course and 30-day post-operative outcomes were collected for each participant. This was reflective of the National Adult Cardiac Surgery Audit (NACSA) dataset (version 5.1.0) collated by the National Institute for Cardiovascular Outcomes Research (NICOR). The dataset is developed by NICOR with clinical direction and strategy provided by the Society for Cardiothoracic Surgeons (SCTS). The NACSA dataset collates known pre-operative risk factors, past medical history and clinical status alongside known and suspected intra-operative and post-operative surgery-related events.

All participants were followed-up for a period of at least 30 days post-operatively or throughout their hospital admission if this extended beyond that. Participants who were re-admitted post-operatively were also followed-up throughout the subsequent hospital admission. This was in line with England's combined Hospital Episode Statistics and Office of National Statistics (HES-ONS) definition of perioperative-associated cause of death and NICOR NACSA datasets (195). The primary endpoint is adverse events as defined below within the 30-day post-operative period or within the same hospital admission.

Adverse Post-Operative Events

Adverse post-operative events collected were: Death within 30 days (or same admission) of surgery, cardiovascular events and neurological events. The cardiovascular events defined are as new-onset post-operative angina, myocardial infarction (MI), or new graft failure. The

neurological complications are defined as new post-operative transient ischaemic attack (TIA) or stroke. All events are associated with plaque rupture events. These definitions of adverse events are derived from the current NASCA NICOR dataset definitions of adult cardiac surgery-related adverse events.

All pseudo-anonymized electronic data was kept securely on Liverpool John Moores University servers. When the complete dataset had been produced and pseudo-anonymized, the accuracy and validity of this data was subsequently independently verified by a second member of the research team.

Statistical Analysis

All data were presented as mean with standard deviation together with median. If there was a large difference in mean and median, this suggests that the data may be skewed. The range for each variable was also presented to demonstrate the variance within participants. The scatterplots are presented in *appendix* one. All data was first assessed for the "level" of data. Nominal data was assessed using cross-tabulations including Chi Squared and Fischers exact test. Ordinal data such as ejection fraction typically used non-parametric statistical analysis. Interval continuous data such as BMI were assessed using correlative analysis. The kurtosis and skewness was measured using a D' Agostino-Pearson goodness-of-fit test. If there were two variables and the data was normality distributed, a t test was used. If the data was non-normally distributed, the non-parametric equivalent was used. If there were more than two variables, a one-way ANOVA was used. If the data was non-normally distributed, the non-parametric equivalent was used. If the data was binary, a Chi-squared and Fischers exact test were used (95% confidence

interval). Whereas the Chi-squared test assumes a large dataset and therefore provides an approximation, the fischers exact test provides an exact value and is therefore more powerful in datasets with less than five counts (134). If the data was continuous, normality testing was first performed using D' Agostino-Pearson goodness-of-fit testing. A Pearson correlation was used is the data was normally distributed. If it was non-normally distributed, a Spearman correlation was used. Statistical significance is defined as a $P<0.05$.

Study Power Calculation

Assuming 5% significance, and given the difference in AEs we observed of 12.5%, we have a power of 50.2% to draw the conclusions in this thesis. A future, fully powered study, is needed to confirm the results in this work.

6.10. Results

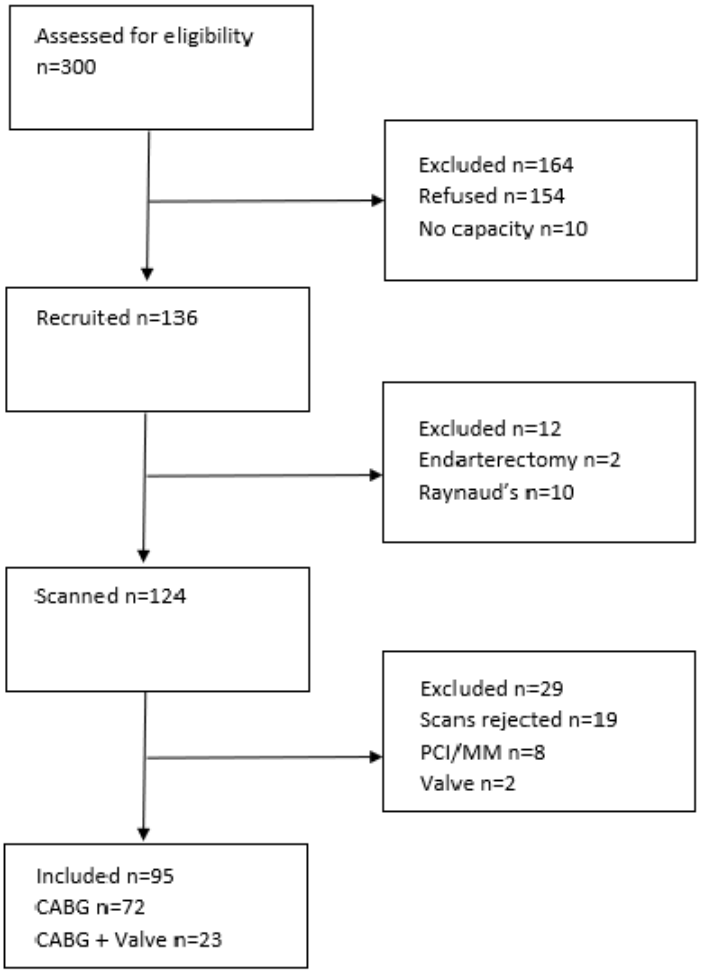
The results were divided into four sections. First, a description of the characteristics of the population. Second, a comparison of participants who did, and did not have post-operative AEs. Third, a comparison of participants dichotomized based on the median CAR%. Finally, logistic regression analysis to investigate if EuroSCORE II and CAR% predict post-operative AEs in this group.

Consort Diagram

A CONSORT diagram of participants assessed, approached and recruited in to this study. As all follow-up used patient medical records, there was no loss to follow-up. A diagram describing follow-up is therefore not included. In this study, only approximately 45% of participants in the

eligible pool were recruited. This is due to a lack of availability in outpatient clinics within the trust. This is detailed further within the discussion section of this chapter.

***Figure 8*)** A CONSORT diagram of recruitment process, enrolment and outcomes.



Adverse Events Group Characteristics

Ten participants experienced AEs post-operatively (as defined in the "*Methods*" section of this chapter). The participant characteristics are presented in ***Table 13 and 14***. There was no statistically significant difference (including age, BMI, symptom severity or clinical risk score) in patients that did and did not have AEs. There was no statistically significant difference in baseline diameter or CAR% in these groups.

***Table* 13)** Group characteristics of participants when grouped according to the presence or absence of Adverse Events. N=95. Independent samples T Test.

	Total Group (95)			**Adverse Events**						
				Yes (10)			**No** (85)			
	Mean/ Median (IQR)	**Range**	**SD**	**Mean/ Median (IQR)**	**Range**	**SD**	**Mean/ Median (IQR)**	**Range**	**SD**	**P Value**
Age (years)	69	45 (41-86)	10	70	33 (50-83)	10	68	45 (41-86)	10	0.73
BMI (kg/m^2)	29.62	22.41 (20.53-49.51)	4.92	29.01	19.32 IQR: 9.62 (23.32 - 42.12)	6.74	29.75	22.32 (20.74-43.73)	5.43	0.46

NYHA Status[1] (1-4)	2	3 (1-4)	1.4	2	3 (1-3)	-	2	3 (1-3)	-	0.13
CCS Status[2] (1-4)	2	IQR 3 (0-4)	1.3	1	3 (1-3)	-	2	4 (0-4)	-	0.15
EuroSCORE II (%)	1.24	6.72 (0.31-7.30)	1.17	2.27	6.53 (0.53-7.03)	2.18	2.86	2.72 (0.31-6.57)	11.13	0.91

[1]: New York Heart Association of Heart Failure symptom classification

[2]: Canadian Cardiology Society Angina classification

Table 14) Carotid artery diameter measurements in the total group as well as those in AE and non-AE groups. N=95. Independent samples T Test performed.

				Adverse Events						
	Total Group (95)			**Yes** (10)			**No** (85)			
	Mean/ Median (IQR)	**Range**	**SD**	**Mean/Median (IQR)**	**Range**	**SD**	**Mean/ Median (IQR)**	**Range**	**SD**	**P Value**
Baseline (cm)	0.80	0.61 (0.52-1.15)	1.10	0.77	0.29 (0.59-0.88)	0.09	0.79	0.61 (0.54-1.14)	0.11	0.72
Diameter[1] (cm)	0.79	0.66 (0.52-1.18)	0.11	0.78	0.25 (0.61-0.86)	0.08	0.80	0.25 (0.52-1.18)	0.11	0.58
CAR (%)	1.93	16.12 IQR 2.88 (-6.18– 9.31)	2.97	0.68	8.63 (-3.87-4.76)	2.49	0.74	16.12 (-6.81-9.31)	2.97	0.12

[1:] Average change in diameter across the CPT

The presence of risk factors and disease severity in AE and non-AE groups are presented in (***Table 15*** and ***16*** respectively). There was no statistically significant difference in measures of disease severity or risk factors in the AE and non-AE groups (***Table 15***). There was a statistically significant difference in the ratio of patients who had on-pump vs off-pump surgery in the AE vs non-AE groups (***Table 16***). A larger proportion of patients who had AEs had off-pump CABG surgery. (**X^2_a: 0.02 F_b: 0.04**).

***Table* 15)** Family History and known risk factors in AE and non-AE groups. Fischers exact and Chi Squared test.

	Adverse Events				
	Yes (10)		**No** (85)		
	Positive (count)	**Negative (count)**	**Positive (count)**	**Negative (count)**	**P Value**
Gender	Female: 1 (10%)	Male: 9 (90%)	Female: 17 (20%)	Male: 68 (80%)	X^2_a:0.45 F_b: 0.68
Family history of CVD	7	3	3	50	X^2_a:0.11 F_b: 0.10
Hypercholesteremia	7	3	59	27	X^2_a:>0.99 F_b: 0.93
Peripheral vessel disease	2	8	10	76	X^2_a:0.16 F_b: 0.20
Previous MI	4	6	26	57	X^2_a:0.58 F_b: 0.72
Previous PCI	2	8	19	67	X^2_a:0.80 F_b:>0.99

Diabetes	1	9	30	56	X^2_a:0.11 F_b:0.16
Pulmonary disease	2	8	9	77	X^2_a:0.31 F_b: 0.32
Neurological dysfunction	2	8	7	79	X^2_a:0.22 F_b: 0.24

***Table* 16)** Effect of disease severity in adverse event and non-AE groups. If the variable is binary a Chi Squared and fischers exact test is used. If there are two variables, a t test is used. If there are more than 2 variables, a one-way ANOVA is used.

	Adverse Events		**P Value**
	Yes (10) (count)	**No** (85) (count)	
Coronary vessel disease[1]			P: 0.10
One vessel >50%	5	27	
Two vessels >50%	0	7	
Three vessels >50%	5	51	
Left main stem disease[1]			X^2_a: 0.21 F_b: 0.32
No	7	53	
Yes	3	33	
Ejection fraction category[3]			P: 0.30
Good (LVEF >50%)	7	70	
Fair (LVEF 30-50%)	1	14	
Poor (LVEF <30%)	2	2	
Number of valves repaired/replaced			P: 0.18
0	8	64	
1	1	15	
2	1	5	
Cardiopulmonary bypass			**X^2_a: 0.02** **F_b: 0.04**
No	8	35	
Yes	2	50	

Operative Urgency Elective Urgent	 7 3	 68 17	**P: 0.12**
Cardiopulmonary Bypass Time	-	-	**P: 0.08**
Cross Clamp Time	-	-	**P: 0.22**
Length of Stay	-	-	**P: 0.03**
ICU Stay	-	-	**P: <0.01**

[1] Diagnosed via angiography, CT or MRI.

[2] Left main stem disease greater than 50%

[3] Ejection fraction categories using NICOR dataset and taken from medical notes.

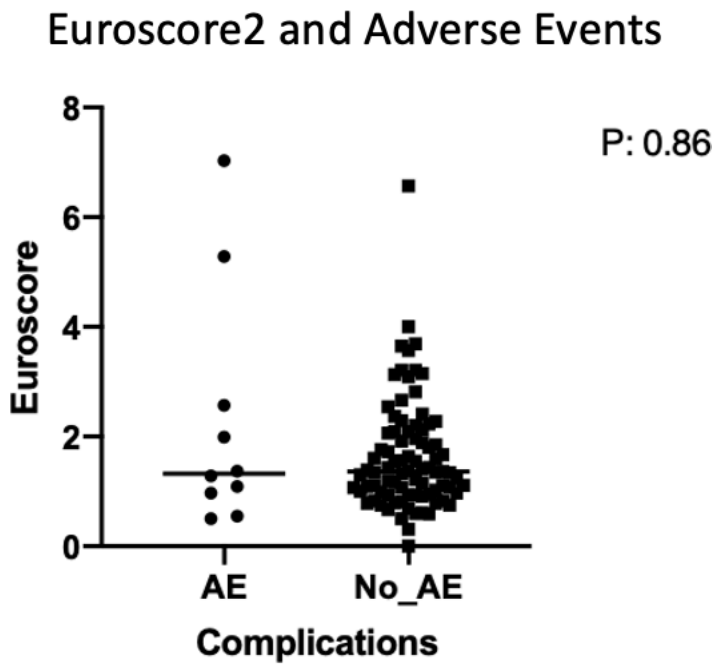
[a:] Chi Squared

[b:] Fischers exact test

Adverse Events and EuroSCORE II

The averaged multivariable risk score (EuroSCORE II) was compared in the AE and non-AE groups. This was presented in ***Figure 9***. There was no statistically significant difference in EuroSCORE II between the AE and non-AE groups.

Figure **9**) A comparison of EuroSCORE II score in participants in AE and non-AE groups. Independent samples t test used. AE is participants that suffered an adverse event as defined in the methods section of this chapter.



Demographics in participants dichotomized according to median CAR%

The primary hypothesis of this study was that patients who have post-operative AEs have significantly poorer endothelial function. The participant were therefore dichotomized according to their CAR% to the CPT. There are currently no studies that have characterized what is a normal and healthy responses versus a response demonstrating significant endothelial function. The participants were dichotomized in to the top and bottom 50% according to median CAR%. The data was skewed with a large range, therefore both mean and median are provided. The effect of group characteristics, and measures of carotid artery diameter on CAR% were investigated. The participants were first grouped into those greater than and less than median CAR%. Median CAR% was 1.96%. This data was presented in ***Table 17***. There was no statistically significant difference in group characteristics when participants are grouped according to median CAR% response. There was a statistically significant difference in EuroSCORE II score **(P: 0.01)** and CAR% **(P: <0.01)** in these groups.

***Table* 17)** Characteristics of participants when separated into those with greater than, and less than median CAR% (1.96%)

	Median CAR% (1.96%)						
	<median			**>median**			
	Mean	**Range**	**SD**	**Mean**	**Range**	**SD**	**P Value**
Age (years)	69.32	39.12 (47.63-86.73)	8.43	68.41	44.41 (41.83-85.42)	10.22	0.61
BMI (kg/m^2)	29.32	20.92 (20.51-41.43)	4.88	29.91	20.82 (22.21-42.91)	5.12	0.50
NYHA status$_1$ (1-4)	1	3 (1-3)	-	2	3 (1-3)	-	0.57
CCS status$_2$ (1-4)	2	4 (0-4)	-	2	4 (0-4)	-	0.51

EuroSCORE II (%)	2.00	6.72 (0.31-7.03)	1.45	1.42	3.53 (0.51-4.00)	1.42	**0.01**
Baseline (cm)	0.79	0.53 (0.54-1.07)	0.11	0.78	0.55 (0.59-1.15)	0.11	0.73
Diameter[3] (cm)	0.80	0.54 (0.52-1.06)	0.11	0.80	0.57 (0.61-1.18)	0.12	0.92
CAR (%)	-0.50	8.79 (-0.68-1.93)	2.27	3.85	8.11 (1.98-9.31)	1.74	**<0.01**

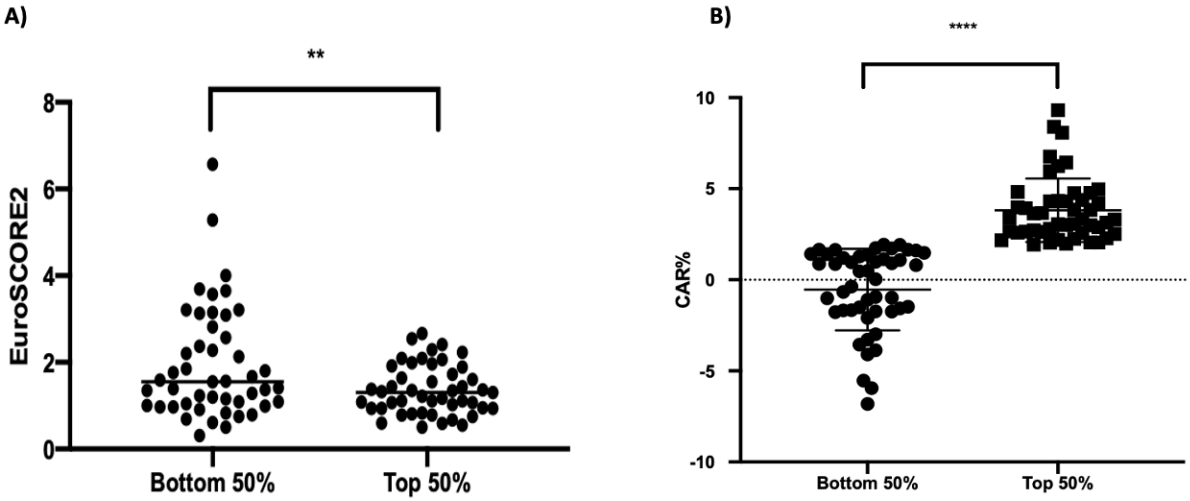
[1]: New York Heart Association of Heart Failure symptom classification

[2]: Canadian Cardiology Society Angina classification

[3:] Average change in diameter across the CPT

EuroSCORE II and CAR% in participants dichotomized according to CAR%.

Figure 10) A comparison of EuroSCORE II and CAR% in participants when they are dichotomized according to their CAR%. **A)** There is a statistically significant difference in EuroSCORE II when participants are dichotomized according to their median CAR% response (p: 0.001). Independent samples t test. **B)** There is a statistically significant in CAR% when participants are dichotomized according to their median CAR% (p:<0.001). Independent samples t test.



The relationship between family history, risk factors and disease severity were then investigated in participants when they are grouped according to median CAR%. This was presented in ***Table 18*** and **19** respectively. There was no statistically significant difference in measures of disease severity or risk factors in participants when grouped according to their median CAR% response.

***Table* 18)** Effect of Family History and known risk factors in those with a CAR% above, and below median CAR%. Chi Squared and Fischers exact test performed.

	CAR				
	Bottom 50%		**Top 50%**		
	Positive (count)	**Negative (count)**	**Positive (count)**	**Negative (count)**	**P Value**
Gender	Female: 12	Male: 16	Female:6	Male: 41	X^2_a: 0.13 F_b: 0.19
Family history of CVD	22	26	21	26	X^2_a: 0.91 F_b: >0.99
Hypercholesteremia	31	17	35	12	X^2_a: 0.30 F_b: 0.40
Peripheral vessel disease	8	40	4	43	X^2_a: 0.23 F_b: 0.36
Previous MI	20	28	13	34	X^2_a: 0.20 F_b: 0.15
Previous PCI	12	36	9	38	X^2_a: 0.50 F_b: 0.62
Diabetes	13	35	18	29	X^2_a: 0.24 F_b: 0.28
Pulmonary disease	6	42	5	42	X^2_a: 0.78 F_b: >0.99
Neurological dysfunction	4	44	5	42	X^2_a: 0.70 F_b: 0.74

***Table* 19)** Effect of disease severity in those with a CAR% above, or below median. If the variable is binary a Chi Squared and fischers exact test is used. If there are two variables, a t test is used. If there are more than 2 variables, a one-way ANOVA is used.

	Median CAR		
	Bottom 50% (count)	**Top 50% (count)**	**P Value**
Coronary vessel disease[1]			P: 0.12
One vessel >50%	21	11	
Two vessels >50%	2	6	
Three vessels >50%	25	30	
Left main stem disease[1]			X^2_a: 0.77 F_b: 0.83
No	31	29	
Yes	17	18	
Ejection fraction category[2]			P: 0.58
Good (LVEF >50%)	37	39	
Fair (LVEF 30-50%)	9	6	
Poor (LVEF <30%)	2	2	
Number of valves repaired/replaced			P: 0.40
0	38	36	
1	5	9	
2	4	2	
Cardiopulmonary bypass			X^2_a: 0.36 F_b: 0.41
No	20	23	
Yes	28	24	

[1] Diagnosed via angiography, CT or MRI.

[2] Left Main Stem Disease greater than 50%

[3] Ejection Fraction categories using NICOR dataset and taken from medical notes.

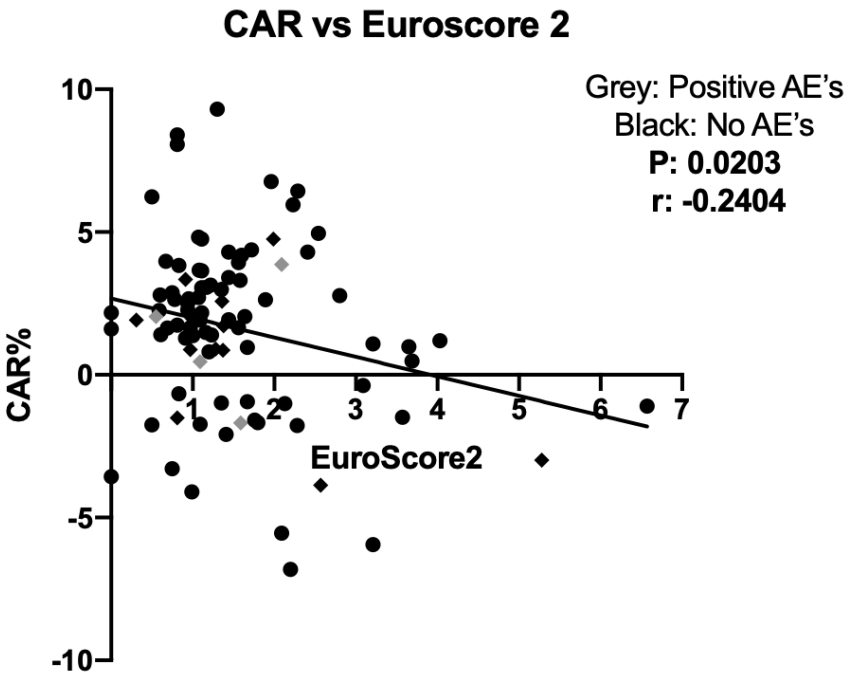
[a]: Chi Squared

[b]: Fischers exact test

CAR% vs EuroSCORE II

The multivariable risk score (EuroSCORE II) on endothelial function (CAR%) were then investigated. EuroSCORE II is the gold-standard score used clinically to predict risk of mortality in patients undergoing cardiac surgery. This was presented in ***Figure 11***. There was a weak inverse correlation between EuroSCORE II and CAR% (**P: 0.011 r: -0.259**)

Figure 11) Relationship between CAR% and EuroSCORE II. "Positive AE" defined as participants who suffered an adverse-event post-operatively. Pearson correlation performed.



Adverse Events in Participants Grouped According to Median CAR%

The number of AEs were then investigated in participants when dichotomized according to median CAR%. This was presented in ***Table 20***. There was no significant difference in the number of AEs in the two groups. It should be noted that there were only a small number of AEs. Chi-Squared value is an approximation and performs more poorly in datasets with less than 5 counts (134). Fischers exact value is therefore also provided.

***Table* 20**) Number of AEs in dichotomized participants. Chi Squared and Fischers exact test performed. A one-tailed analysis was performed with a 95% confidence interval.

Categories	**No AE's (count)**	**AE's (count)**
Top 50%	45	2
Bottom 50%	40	8
P value	**X^2_a:0.05** ***F_b*: 0.09**	

[a] Chi Squared

[b] Fischers Exact test

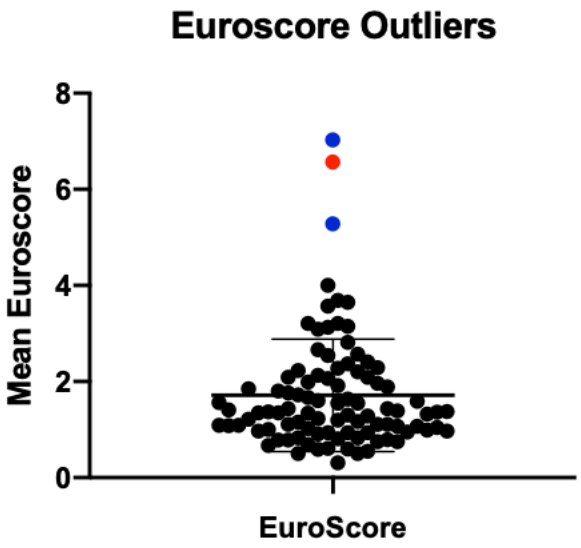
Predictive capacity testing

The presence of outliers was first investigated to normalize the dataset. To investigate the primary and secondary hypotheses. The ability of EuroSCORE II (gold standard) and CAR% to predict post-operative AEs were investigated. This was investigated using non-linear regression analysis. The impact of CAR% on the predictive capacity of EuroSCORE II was then investigated. This was investigated using multivariable logistic regression analysis.

Identifying Outliers

The sum-of-squares calculation (a study of variance within regression analysis) is highly sensitive to outliers (196). A ROUT analysis was undertaken first to identify outliers within the dataset. A ROUT analysis was performed as outliers within non-linear regression analysis is highly sensitive to outliers. Outliers within these datasets can dominate the sum-of-square analysis calculation, thereby skewing both logistic regression and multivariable logistic regression analysis (196). Based on a 1% False Discovery Rate (FDR), 3 outliers were identified. This was presented in ***Figure 12***. These participants had a EuroSCORE II score of 5.28%, 6.57% and 7.03% respectively.

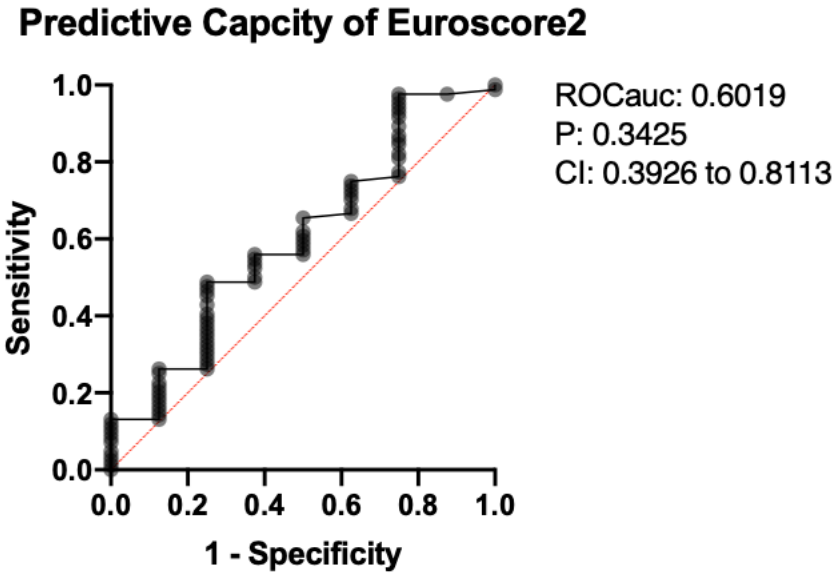
Figure 12) ROUT analysis Investigating the presence of outliers within the dataset. This identified three outliers in which two had AEs (blue) and one did not (red).



Predictive Capacity of EuroSCORE II

The ability of EuroSCORE II to predict risk was then investigated. EuroSCORE II is the current gold standard and is used in clinical practice to predict AEs in cardiac surgery. This was presented in ***Figure* 13**. In this study, EuroSCORE II did not predict risk of AEs.

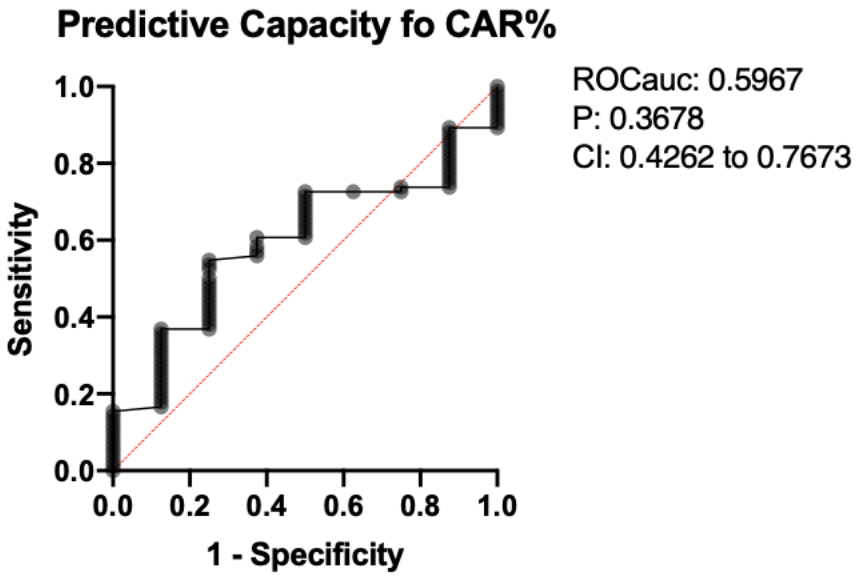
***Figure* 13**) Logistic regression analysis of the ability of Eurscore2 to predict AEs in CABG patients.



Predictive Capacity of CAR%

The ability of CAR% to predict risk was then investigated. This is presented in ***Figure 14***. In this group, CAR% does not predict post-operative AEs.

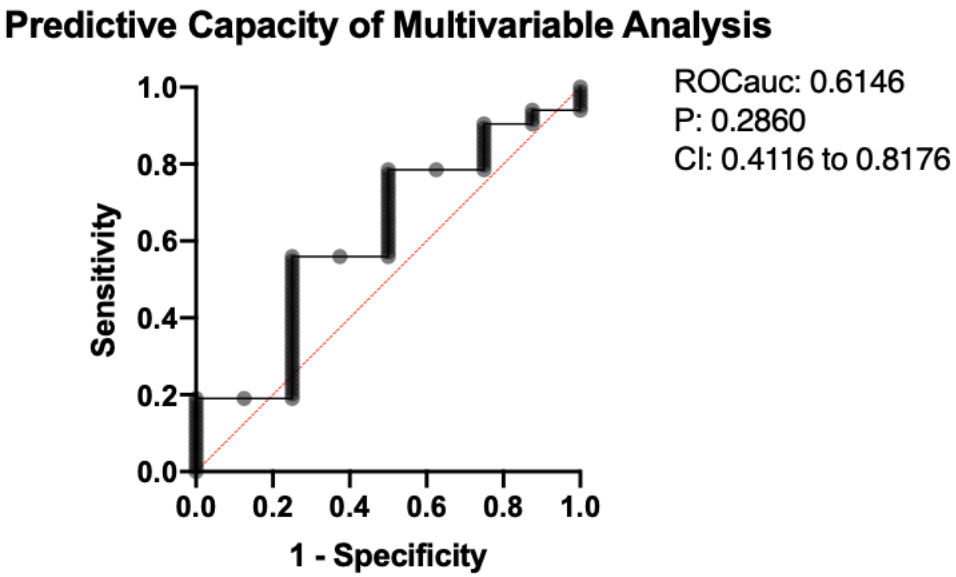
***Figure* 14**) Logistic regression analysis of the ability of CAR% to predict AEs in patients undergoing CABG surgery.



Multiple Logistic Regression Analysis of Combined CAR% and EuroSCORE II

The combined ability of EuroSCORE II (gold standard) and CAR% to predict AEs was then investigated. A multivariable logistic regression analysis method was used. This was presented in ***Figure 15***. EuroSCORE II combined with CAR% did not predict risk in this group.

***Figure* 15**) Multiple logistic regression analysis of the ability of CAR% combined with EuroSCORE II to predict AEs in CABG patients.



6.11. Discussion

Whole Group Characteristics

This study included a small number of patients, therefore this study was first compared to one of the largest multi-site studies involving approximately 32000 participants undergoing CABG surgery between 2012 and 2014 (197). In our study, participants were on average 5.1 years older than that of the comparable study (69 years in our study vs 64 years). Our study had a smaller percentage of participants with 3 vessel disease compared to the larger-scale study (62% in our study vs 65.5%). In this study, 28.4% of participants had a CCS angina score of 0, 32% had a CCS score of 1-2 and 38% had a CCS score of 3-4. Whereas in the comparable study, 0% had a CCS score of 0, 37.5% had a CCS score of 1-2 and 62.5% had a CCS score of 3-4. Taken together, this highlighted the relatively low risk profile of the participants included in this study. This may have been as a result of predominantly recruiting from the outpatient setting.

EuroSCORE II

In this group, EuroSCORE II was not correlated with individual risk factors or disease severity. Similarly, EuroSCORE II did not predict risk of cardiovascular or neurovascular AEs or death in this study. Although EuroSCORE II is a validated risk-prediction tool and is used clinically, there are a number of limitations to using EuroSCORE II to predict post-surgical AEs. First, EuroSCORE II was originally designed to predict operative mortality. However it has also been adopted to predict AEs (198). Only one patient died within this study, therefore EuroSCORE II was correlated with post-operative AEs rather than mortality. A small number of studies have investigated the ability of EuroSCORE II to predict postoperative AEs. Ranjan et al (2019) demonstrated that EuroSCORE

II can predict post-operative MI (ROC_{AUC}:0.854) but is poorly predictive of stroke (ROC_{AUC}: 0.646). This was a larger study of 865 participants with 29 (MI) and 7 (Stroke) events respectively (199). They therefore demonstrate good predictive capacity for MI, but poorer predictive ability for stroke. Similarly, Biancari et al (2012) found EuroSCORE II was poorly predictive of stroke in 1027 patients undergoing isolated CABG (ROC_{AUC}: 0.649) (200). The poor ability of EuroSCORE II to predict cardiovascular AEs, cerebrovascular AEs and death in our group was therefore in-line with previously published research particularly in neurovascular events and would therefore require a large number of events to demonstrate a predictive capacity.

Second, It has been demonstrated previously that EuroSCORE II performs best in moderate risk groups, and performs poorly in low-risk and high-risk individuals (201). In our study, when outliers were removed (ROUT analysis, Q Rate 1%), mean EuroSCORE II score was 1.56% (0.31%-4%). In line with previously published work, a EuroSCORE II score of less than 2% are considered low-risk and a EuroSCORE II score of less than 1% as very low risk (202). This was therefore a low risk group and this finding was in-line with previous studies which have demonstrated that EuroSCORE II performs poorly in lower risk groups (201).

Third, the EuroSCORE II risk tool was first launched in 2011. There have been a considerable number of changes in National Institute for Health and Care Excellence (NICE) guidelines, and an increased move towards PCI in this time (203). Similarly, Lebreton et al (2011) also highlighted the inability for clinicians to specify the surgical procedures undertaken. The EuroSCORE II tool includes the number of procedures undertaken, but not the specific interventions. This is in contrast to the STS tool, in which surgical interventions are specified (168). This may mean the

STS tool allows a more patient-specific approach to risk stratification and tailoring to higher-risk interventions. This may be required to be incorporated into future updates of the EuroSCORE tool.

Finally, EuroSCORE II is a risk prediction tool derived from large datasets and therefore does not allow the personalization of risk prediction or the addition of emerging factors which predict AEs such as endothelial function. Although EuroSCORE II does include a small number of risk factors known to affect endothelial function, this does not include atheroprotective factors such as diet and exercise (204, 205). Taken together, EuroSCORE II did not predict risk in this group. This was in-line with previous studies within the literature. This may provide further evidence for a requirement for further refinement of EuroSCORE II including the ability to specify intervention types as well as the inclusion of emerging risk prediction tools including measures of endothelial function as a surrogate of vascular health.

Carotid Artery Response

Previous studies have demonstrated that CAR% is a strong predictor of disease progression and events in PAD patients (54). However, to our knowledge this was the first study investigating if CAR% predicts AEs in CABG patients. In this group, CAR% did not predict risk in patients undergoing CABG surgery. This may be due to a number of factors.

First, Endothelial function has been demonstrated to predict risk in cardiovascular disease patients. However, measures of endothelial function do not incorporate other factors such as past medical history, active infection, critical preoperative status or a recent history of MI. All of which affect post-operative outcomes indepenbydent of endothelial function (35, 168, 201).

Although these may be influenced by endothelial function, these are not directly incorporated in to measures of endothelial function.

A second key consideration is that CAR% was used as a measure vasomotion as a surrogate of systemic endothelial function/health. Although risk prediction has traditionally relied upon investigating plaque burden within the coronary arteries, and identifying flow limiting lesions, The majority of MIs are caused by non-flow limiting lesions (206). Similarly, a large amount of plaque rupture events are asymptomatic in the short-term but may lead to eventual narrowing (206). This highlights the importance of identifying high-risk plaque that are vulnerable to rupture and are likely to cause ischemia. A number of key markers of high-risk plaque have been identified including a large necrotic core, extensive calcification, and macrophage infiltration (206, 207), alongside emerging novel CT and ultrasound imaging characteristics (208-210) Similarly, a growing body of evidence has found that coronary inflammation measured both systemically (plasma biomarkers) and locally predict risk of AEs (108). Recently, Antoniades et al (2020) demonstrated that coronary inflammation quantified as perivascular fat attenuation index (FAI) improved cardiovascular risk discrimination for all-cause and cardiac mortality when compared to current risk factor analysis (smoking, diabetes), extent of coronary atherosclerosis and high risk plaque features (108, 211). Taken together, this highlights the complex multifactorial nature of plaque rupture events and further highlights the limitations of measuring disease burden or systemic endothelial function as a predictor of AEs.

Third, carotid artery vasomotion is a complex vascular response encompassing the entire length of the artery resulting in a three-dimensional constriction or relaxation in response to stimuli.

Whereas two-dimensional carotid artery ultrasound measures a small representative segment of the entire artery. The quantification of carotid artery response measures the distance between proximal and distal walls of the vessel along a single plane. Although CAR% is relatively simple and non-invasive, it may not have been representative of the complex three-dimensional response along the entire artery. This was not fully investigated within our study or the literature. This was discussed extensively in *Chapter 7* of this thesis. Similarly, CAR% was used as a measure of vasomotion as a surrogate of endothelial function. Vasomotion is reliant upon vascular distensibility. However, it was previously demonstrated that the presence of plaque affects vascular distensibility and vasomotion. No obvious plaque was included in the segment of vessel scanned as part of this study. Giannattasio et al (2001) demonstrated that carotid artery distensibility was significantly lower in both the internal and common carotid artery in the presence of plaque when compared to contralateral plaque-free arteries in patients with hemodynamically significant unilateral atherosclerosis with >75% occlusion (212). Importantly, arterial distensibility was also significantly reduced in the plaque-free common carotid artery if the internal carotid artery has plaque >75% (212). This highlight the impact of atherosclerosis on arterial distensibility both proximal and distal to the plaque site. This may alter the CAR% response in atherosclerotic arteries. Puri et al (2013) demonstrated that coronary atheroma volume was associated with endothelium-dependent vasoreactivity independent of clinical presentation (213). This is a key consideration as patients undergoing CABG surgery have a large variance in the severity and presence of carotid artery atherosclerosis in neurologically asymptomatic CABG patients (214). The alteration of distensibility and therefore vasomotion in the presence of atherosclerosis may therefore mean that CAR% can only be used in patients

without evidence of carotid artery atherosclerosis, although this has not been tested. Taken together, CAR%, did not predict risk in this group. This may be as a result of a number of considerations in endothelial function as an independent risk prediction tool as well as key considerations in the use of CAR% to investigate endothelial function. There are also practical considerations and limitations associated with our study. Although CAR% was not clinically useful as a risk prediction tool in isolation, it may offer a simplistic and non-invasive tool to measure endothelial function which can be incorporated and used along-side tools which measure other factors known to predict AEs such as measures of systemic health and clinical status.

Multivariable Logistic Regression Analysis

EuroSCORE II alone and CAR% alone did not predict risk. It was then investigated if CAR% as a measure of endothelial function added to the predictive ability of EuroSCORE II. When a multivariable logistic regression analysis was performed, EuroSCORE II combined with CAR% did not predict risk of AEs. However, this did marginally increase the ROCauc. This may be suggestive of an improvement. Although in this study, it is not possible to conclusively investigate this. As others have investigated CAR_{AUC} and $CAR_{90,}$ and demonstrated that these predict risk (115). These were also investigated. Both CAR_{AUC} and CAR_{90} do not predict risk in this group (r: 0.364 and r:0.454 respectively). Data not shown. This lack of correlation may be due to a number of factors.

First, there was a weak inverse correlation between EuroSCORE II score and CAR% with patients at the highest risk of surgical AEs also having the poorest endothelial function. The correlation between EuroSCORE II and CAR% may explain the small improvement in predictive capacity as both EuroSCORE II and CAR% did not predict risk. EuroSCORE II incorporates a small number of

risk factors which affect endothelial function such as increased age and diabetic status (135), as well as factors that may be a marker of endothelial dysfunction such as disease severity, symptom severity and presence of PAD (14, 54). The combination of both EuroSCORE II and CAR% may be disproportionately weighting risk factors associated with endothelial (dys)function above other key factors. To overcome this, in future CAR% may be treated as a multivariable analysis of known and suspected factors which affect endothelial function and can then be considered in the weighting of risk stratification tools. However, the weighting of individual variables within the EuroSCORE II tool are not published. It should also be noted that there are only a small number of events within this study. A larger study, including a higher incidence of AEs would first have to be investigated and would be more able to identify moderately predictive tools.

Medical Management/Percutaneous Coronary Intervention

In this study, ten participants who were first identified as requiring CABG surgery did not undergo CABG surgery. These patients were thought to have an unacceptably high risk of AEs or had small focal disease and therefore underwent conservative management. In our study, two of the three patients who did not undergo CABG surgery due to the high-risk nature demonstrated a negative CAR%. Whereas in patients who underwent conservative management (PCI or Medical Management (MM)) due to the low severity or sub-clinical coronary artery disease, six of the seven patients demonstrated carotid artery dilation. It should be noted that in the medical PCI/MM group, the participant with the poorest endothelial function (CAR% -4.57%) had small focal disease and therefore opted for PCI. This patient was therefore comparatively very low risk. This was an interesting finding suggesting that CAR% may be in agreement with clinical decision

making in this group, although this is underpowered. The inability to follow-up these patients over a prolonged period of time, and an inability to perform hazard ratio analysis limits conclusions in this group, as it is unknown if this was the correct management for these individuals.

Data quality

A key consideration in any research project is the quality of the data. In this study, there are a number of opportunities for limitations in data quality.

Scan acquisition

A key consideration in clinical studies involving imaging is in the quality and standardization of imagine acquisition and processing. When utilizing the carotid artery in imaging research, it is key to try to standardize scan acquisition. In this study, the area distal to the carotid bulb was utilized. An area adjacent to, but not including the carotid bulb is utilized. The largest area of Region Of Interest was included to ensure scans were representative of the entire arterial segment imaged.

Scan Analysis

All scans were interpreted blinded to ensure there was no bias in demonstrating vasoconstriction in patients who were at an increased operative risk. All scans were also analysed independently by two researchers to ensure quality in scan. As all scans were only undertaken once, the variance in scanning could unfortunately not be measured.

Clinical Data

All data collected was taken directly from the patients clinical records. The use of paperless medical notes means that there were no "missing" datapoints during the follow-up period. The accuracy of the data was also ensured by a second member of the research team. A randomly selected sample of ~30 participants were selected and the data input on the follow-up spreadsheet was checked against the medical records. This was to ensure the accuracy and validity of data extraction. This was undertaken by a second member of the research team independently. As all files are stored electronically, there was no missing data in this study.

Study Limitations

There are a number of limitations or considerations associated with our study. Firstly, this study involved 95 participants and ten AEs. In logistical regression analysis a further three participants (one AE) were removed due to being outliers. Although the number of events per variable were acceptable for both linear regression and multivariable analysis, this would likely have been underpowered to identify moderately predictive tools. The number of participants is also lower than the predicted number of participants required. Due to limitations in recruitment from outpatient services, recruitment was expanded to include inpatients. In retrospect, recruitment should have included both outpatient and inpatient services from the beginning. Similarly, recruitment may have been opened up to multiple study sites to further improve recruitment. This may have also increased the variance in demographics and risk profile of participants involved in the trial making this more representative of moderate and higher risk patients.

A second limitation is that EuroSCORE II alone was used. Although EuroSCORE II is current clinical practice within the UK, there are a relatively small number of variables included in this that are

known to affect endothelial function such as age and diabetic status. The US STS risk tool investigates a larger number of variables which are known or suspected to affect endothelial function such as exercise tolerance. The STS score could have been compared to both EuroSCORE II and CAR% as well as investigating if CAR% improved the predictive capacity of STS. However, this was unfeasible as a number of the variables included in the STS score are not routinely recorded in the UK. A questionnaire could have also been included which records both athero-protective (eg exercise) and pro-atherosclerotic (smoking, obesity) risk factors to gain a more complete picture of accumulative risk factor profile on endothelial function.

A third consideration is the effect of menopause on female endothelial function. It has been demonstrated previously that there is a reduction in endothelial function in post-menopausal women. It is thought that this is as a result of a loss of endothelially-protective estrogen. There may also be an improvement in endothelial function associated with hormone replacement therapy. A limitation of this study was that, although women were included in the study, we did not control for this as a confounding variable. This was also not controlled in previous studies which have demonstrated the predictive capacity of endothelial function. However, a larger study involving a larger number of participants would allow subset analysis including pre-menopausal women, post-menopausal women and post-menopausal women receiving hormone replacement therapy.

A fourth consideration is the associated between endothelial function and atrial fibrillation. It has previously been reported that poor endothelial function is associated with an 11% increased absolute risk and a 5.8-fold relative risk of atrial fibrillation (215). It is thought this is caused by a

number of inter-related mechanisms including altered hemodynamics, altered shear stress affecting vascular endothelial cells, a reduced nitric oxide bioavailablilty, an increased reactive oxygen species, inflammation and abnormalities in the rennin-angiotensin axis (215). AF has also been associated with an increased risk of adverse events (216, 217). This therefore makes the increased risk of AF associated with poor endothelial function and therefore the increased risk of adverse events a potential confounding variable. In our study we included patients that are currently in AF. However, we did not consider AF a post-operative adverse event. It was thought that, as a significant number of CABG patients are in AF. It was key that, if CAR% was able to predict risk, it should do so in a clinically representative sample of CABG patients.

A fifth consideration was the inability to standardize the time of testing. It has been demonstrated previously that point in the circadian rhythm (218) and menstrual cycle (219) affect endothelial function. It was not possible to standardize the time of testing, point in the menstrual cycle or peri/post-menopause when undertaking testing in clinical populations recruited directly from NHS sites. Similarly, as patients were typically tested within a short time period after diagnosis. This may mean that patients were already under a considerable amount of stress and therefore this may alter SNS response to the CPT. However again, it was impractical to standardize testing after a defined period post-diagnosis. This test has also been previously demonstrated previously to predict AEs and outcomes in PAD patients (54). It was therefore key that if CAR% does improve risk prediction, it must do so in a clinical environment and in clinical populations.

7. Chapter 7: Thesis Conclusions and Future Work

7.1. Chapter introduction

This chapter summarizes the findings of this as well as the impact of this thesis on the research literature. This further explores key areas for development as well as key steps that have to be taken before this method may be used in wider research or in clinical practice.

7.2. Study Summary

This thesis incorporated two studies. The first was a mechanistic study investigating the degree to which peripheral blood pressure was related to carotid artery diameter change during the CAR% test. This study demonstrated that there was no correlation between blood pressure response and CAR%, supporting the hypothesis that CAR% is not predominantly driven by transmural pressure. These findings therefore added further evidence to the validity of CAR% as a measure of endothelial function. The second study investigated the ability of CAR% to predict AEs within 30-days of CABG surgery. The results demonstrated that EuroSCORE II and CAR% alone did not predict post-operative AEs. However, when adding CAR% to the EuroSCORE II, the predictive capacity may have marginally improved, although this was not predictive of AEs. This may suggest a benefit in incorporating CAR% into risk prediction tools. A caveat to this is the low sample size and event numbers within this study. We cannot conclusively investigate the predictive capacity of the CAR% using this study due to the number of events. This therefore requires further work including larger studies including more patients and therefore more AEs.

The present chapter provides an in-depth discussion of the implications of these findings, and key areas that require further investigation. The implications are presented in four sections: one.

physiological regulation of vasomotion and CAR%. Two, future work in scan acquisition. Three, practical considerations for clinical integration and clinical implications of CAR%. Four future work and practical considerations and section five brings together the findings of this thesis alongside the previous findings within the literature and discussed the incorporation of CAR% and the implications on clinical practice.

Thesis strengths and weaknesses

7.3. Study Strengths and weaknesses

The individual strengths and weaknesses of each study have been discussed within each study. This section will discuss the strengths and weaknesses of the thesis as a whole.

Strengths

This thesis presents the findings from two distinct but inter-related studies. Combined, they provide a novel insight in to carotid artery endothelial function in both health and disease. The aim of the first study was to better understand the mechanism that regulates the vasomotive response to CAR%. The second study investigated endothelial function as a method to predict risk in central arterial disease patients undergoing CABG surgery.

A strength of this thesis is that it included both a mechanistic study to better understand the underlying mechanisms which drives a response and second a study which practically investigates the clinical utility of this tool, alongside a study in clinical populations. This first study added validity to the use of CAR% as a measure of endothelial function whilst also informing on the practicalities of undertaking this research in a more easily controlled University environment

before this was transitioned in to a clinical environment. It gave further opportunities to develop ultrasound techniques as well as problem-solving outside of a busy clinical environment.

A second strength is in the robustness of the data collected. In study 2, the data was checked for accuracy by a second independent researcher. All scans were analysed blinded and checked independently by a second researcher. Although these studies did not reach the required number of participants, considerable effort was made to ensure data quality of studies individually and in the thesis as a whole.

Weaknesses

A fundamental limitation in investigating CAR% as a surrogate of endothelial function is that the mechanisms regulating vasomotion are incompletely understood. As discussed within the background of this thesis, vasomotion is regulated through inter-related mechanisms. CAR% is used as a surrogate of endothelial function and although it has been demonstrated previously that CAR% is significantly driven by endothelial function and that vasoconstriction in response to SNS stimulus is a marker of endothelial dysfunction, the influence each inter-related mechanism has on vasomotion is incompletely understood. If CAR% is going to be used as a surrogate measure of endothelial function, it is key to elucidate the degree to which endothelial function regulates vasomotion. However, this remains challenging because the mechanism of vasomotion is regulated by a complex relationship of inter-related mechanisms.

Similarly, although study 1 provided further evidence that CAR% is predominantly driven by endothelially-mediated mechanisms, this study was conducted in just men aged 30-60. Whereas study 2 included both men and women and the average age was 69 years old. As part of the

effort to improve the understanding around CAR%, it is important that the impact of gender is understood in different age-ranges. This is important as it is known that women develop CAD later than men, but that this is associated with increased severity (147). This may be as a result of a loss of the protective effects of estrogen on endothelial function (147). The protective effects of hormone replacement therapy have also been previously demonstrated. Although this is not known (220).

A third limitation is both of these studies is the use of a controlled breathing protocol, rather than using a isocapnic CPT protocol. It has been recently demonstrated that hypocapnia has a profound effect on carotid artery diameter and is therefore a significant confounding variable (221). In this study, as well as in other studies, a controlled breathing protocol was undertaken where participants are coached to breathe normally throughout the research protocol. However Tymko et al (2017) recently demonstrated that the common carotid artery dilated to a greater extent during isocapnic (controlled CO_2) CPT when compared to poikilocapnic (uncontrolled CO_2) CPT (221). They suggest that this is as a result of the common carotid arteries inherent sensitivity to CO_2. This highlights a potential confounding variable within the project as well as other projects measuring carotid artery response to the CPT. This is discussed further within the "future work" chapter of this thesis.

A fourth limitation is the lack of research in to the effects of prehypertensive and hypertensive participants. In study one, we attempted to recruit pre-hypertensive and hypertensive participants to investigate the relationship between central artery endothelial function and hypertension. This is important as there is a correlation between peripheral arterial endothelial

function and hypertension (222, 223) and chronic hypertension may be at least in part as a result of loss of peripheral vascular function. Hypertension is also common in CAD and CABG patients (224).

A fifth limitation is a lack of understanding the effect smoking has on endothelial function in both clinical groups and healthy participants. It has previously been demonstrated that smoking negatively effects endothelial function (225). The smoking status of participants was therefore recorded prior to testing for each study. This was recording in study one using a health style questionnaire and in study two using the patients' medical records. In study one, none of the participants admitted to smoking. In study two, the smoking status and pack years was recorded. However, as there was a limited number of participants, sub-group analysis including smoker's vs non-smokers could not be undertaken. This is a limitation of these studies and the effect of smoking on CAR% in both healthy participants and in patients undergoing CABG surgery is a potential confounding variable and needs further investigation.

7.4. Physiology of CAR; relation with future work

Carotid artery vasomotion was measured as a surrogate of endothelial function. Arterial vasomotion is regulated by a number of inter-related mechanisms. These can be broadly categorized into endothelium-dependent and endothelium-independent mechanisms.

Endothelium-dependent mechanisms of vasomotion

Endothelium-dependent mechanisms of vasomotion are controlled by two inter-related systems; neuronal and metabolic mechanisms.

Neuronal-mediated mechanisms

The SNS is a key regulator of vascular tone, this was investigated in denervated, and regional reinnervated segments of tissue in cardiac transplant patients. There was a larger increase in flow in response to sympathetic stimulation in reinnervated tissue when compared to denervated tissue. This suggested that adrenergic mechanisms regulated tone to a greater extent than metabolic mechanisms during sympathetic stimulation (14). Neuronal regulation occurs through a complex competitive relationship between dilatory and constrictive stimuli driven by α- and β-adrenoreceptors on the endothelium and tunica media (14). This was reviewed extensively in the "background" chapter as well as in ***Figure* 1** in this thesis. The integrity and function of the endothelium are both key to a normal dilatory response following SNS stimulus (14). The endothelium is the key driver of vasomotion through down-stream control of vascular tone through Vascular Smooth Muscle Cells (VSMC's). Indeed, the loss of integrity and therefore function of the endothelium was typified in those at risk of cardiovascular disease and those with established CVD (14). The loss of function of the endothelium was identified via abnormal truncated responses or paradoxical constriction in response to SNS stimulation including the CPT. This constriction response was predictive of risk in cardiovascular disease and cardiovascular events in CVD patients (14, 42, 109). This highlights the key role neuronal control plays in vasomotion.

Metabolic-mediated mechanisms

Metabolic mechanisms also play a crucial stimulus for endothelial-mediated vasomotion. It is technically challenging to distinguish metabolic from neuronal mechanisms in-vivo. The metabolic regulation of vascular tone has been investigated more thoroughly in cardiac tissue

than in cerebral blood flow (CBF). A local increase in myocardial metabolism leads to a decrease in microvascular resistance, which causes an increase in coronary blood flow to meet the increased metabolic demand. In response to the increase in blood flow, the elevation in shear-stress drives eNOS expression and further SMC-mediated vasodilation. Krivokapich et al (1989) demonstrated that a 2.8-fold increase in myocardial work was associated with a 2.2-fold increase in myocardial perfusion. Importantly, the same group demonstrated a requirement-to-perfusion mismatch in patients with CAD (138). This pattern has been duplicated in a subsequent study by the same group (138, 226). This was extensively reviewed within the "Background" section of this thesis. Metabolic-mediated vasomotion is likely to also play a role in cerebrovascular vasomotion, particularly via the carotid arteries. However, the extent to which metabolic drive controls carotid artery vasomotion is unknown.

The partial pressure of CO_2 has been investigated as a surrogate of cerebral metabolism and driver of SS. Changes in end-tidal CO_2 have been demonstrated to be potent stimulants for intra- and extra-cranial vasomotion (227, 228). This increase in end tidal CO_2 was related to dose-dependent changes in carotid artery diameter, likely driven through SS (145).

This highlighted cerebral perfusion's sensitivity to changes in the partial pressure of arterial blood CO_2 (P_aCO_2). An increased P_aCO_2 (hypercapnia) causes a decrease in cerebrovascular resistance and an increase in cerebrovascular blood flow. In contrast, hypocapnia causes an increase in cerebrovascular resistance and decreased CBF. In addition, an increased P_aCO_2 is associated with a shear-stress mediated carotid artery dilation (145). Tymho et al (2017) investigated the effect of CO_2 on carotid artery response to the CPT. They found that the common carotid artery dilated

to a greater extent during an isocapnic CPT (controlled CO_2) when compared to a poikilocapnic CPT (uncontrolled CO_2) (221). They suggest that the mechanism may be due to an inherent sensitivity of the common carotid artery (CCA) to a reduction in partial pressure of end-tidal CO2 ($P_{et}CO_2$) which resulted in an attenuation in blood flow and/or SS response during poikilocapnic CPT (221). This represented a key potential driver of CAR% throughout the CPT. However, end-tidal CO_2 ($P_{et}CO_2$) was not controlled for in our studies. The participants were provided clear breathing instructions and no participants appeared to have hyperventilated throughout testing. This was in accordance with previously published research (49, 54, 229). It is therefore unlikely that P_aCO_2 significantly contributed to the CAR% in either of our studies. However, the impact of P_aCO_2 and therefore SS however remains a potential confounding variable when measuring CAR%. The requirement for a dynamic end-tidal forcing (DEF) system required for controlling P_aCO_2 would limit the use of the CAR% test to research laboratories or specially designed clinical rooms, rather than the point-of-care testing portable ultrasound would allow. A study investigating the impact of a controlled breathing protocol (54, 130, 229) vs dynamic end tidal forcing-controlled (221) P_aCO_2 is key. This would identify the best method to control SS-mediated vasomotion and allow researchers to control this as a potential confounding variable.

The CPT was also associated with a significant increase in blood pressure (BP) which may have affected CAR% (230). This increase in blood pressure lead to an increased SS and transmural pressure. This increase is a potential significant driver of response to CPT. We therefore investigated the relationship between CAR% and the blood pressure response in healthy individuals. We found that the carotid artery diameter response precedes that of the BP response, that there was no correlation between CAR% and BP response, and that there were

comparable BP responses in participants who “dilate” and “constrict” in response to the CPT (130). Taken together, this provides evidence that CAR% was not significantly driven by the increase in BP associated with the CPT. Although it is technically challenging to investigate CAR% in the absence of a blood pressure response. Monahan et al (2013) used α- and β-adrenoreceptor agonists to blunt BP response to the CPT. They found this also abolished vasomotion in the LAD in response to the CPT (139). Similarly, Van Mil et al (2018) reported α-adrenoreceptor blockade blunted BP response and abolished carotid and coronary artery response to the CPT. This highlighted the technical difficulty in investigating CAR% in the absence of a BP response as both BP response and CAR% are adrenoreceptor-dependent (139).

The fact that reinnervated cardiac segments (neuronal and metabolic mechanisms) dilated to a greater degree than de-innervated segments (metabolic mechanisms only). Taken together with the fact that adrenoreceptor blockade abolished carotid and coronary artery vasomotion in response to the CPT suggests neuronal mechanisms are key to vasomotion. Our study suggested that the increase in BP (and therefore SS) associated with the CPT was not a significant driver of CAR%. This added further weight to the theory that CAR% is predominantly endothelial-driven regulated by neuronal mechanisms. However, the profound effect metabolic mechanisms have on carotid artery vasomotion suggest that this may have been a key contributor to CAR%, particularly in hypocapniac CPT. This remains a key potential confounding variable and therefore requires further investigation before CAR% can be used clinically. The precise impact both neuronal and metabolic mechanisms have on CAR% remain technically challenging to elucidate.

Endothelium-independent vasomotion

The ability of VSMC's to dilate is a key component of vasomotion down-stream of the endothelium. Therefore, the vasodilatory response was reliant upon both an intact and functional endothelium as well as arterial responsiveness to Nitric Oxide (NO). Nitroglycerin acts as an endothelium-independent NO donor, and nitroglycerin-induced vasodilation (NID) is used as a measure of endothelium-independent vasodilation. A reduction in endothelium-independent vasodilation has been demonstrated in patients at increased risk of CVD (42, 231, 232) as well as in healthy aging (82, 233). NID has been shown to be impaired in participants with cardiovascular risk factors indicating VSMC dysfunction (234, 235). Importantly NID dysfunction was associated with a higher incidence of cardiovascular events and has been used as a prognostic marker in clinical groups (234, 235). This highlighted the importance of endothelium-independent vasodilation in CAD. The mechanisms by which endothelium-independent vasodilation are inhibited in cardiovascular disease can be separated into structural and functional alterations.

Structural Alterations

The structural alterations affect vasomotion up-stream of a normally functioning endothelium. This is associated with an increase in the connective tissue matrix associated with an increased intima-medial thickness (IMT). This may limit relaxation and increase proliferation of VSMC's which causes a relative decrease in NO availability derived from nitroglycerin (236). This increased IMT is thought to be as a result of progressive calcification, elastic fibre degeneration and increased collagen content alongside VSCM proliferation (237). This results in an increased rigidity within the artery and reduced vasomotion independent of a functioning endothelium. This is supported by clinical studies which have shown that an increased IMT is associated with

cardiovascular risk and poorer outcomes (238-240). However, IMT was not measured in this study. It was difficult to maintain the lines of Pignoli (IMT visualized using ultrasound) whilst scanning in this group. This was due to the practical challenges of scanning overweight participants. In study two, 40 participants were overweight (BMI 25-30) and 39 were obese (BMI 30+). This study investigated carotid artery function, rather than structure. A consistent ROI was therefore selected rather than prioritising maintaining the lines of Pignoli. It was not possible to identify patients with an increased IMT and therefore altered structure. A number of studies have demonstrated an inverse correlation between IMT and vascular function including brachial (38, 157, 241) and femoral arteries (157, 242, 243). Studies which investigated the relationship between carotid IMT and function via FMD have demonstrated mixed results. Some studies have found an inverse correlation between IMT and function, whereas others have demonstrated a positive correlation (244-248). This may be as a result of variances in methodology including artery used in the FMD and population group. In future studies, IMT could be measured in all participants allowing a relatively simple measure of carotid artery structure. This would also identify patients with increased IMT and therefore structural alterations which may affect CAR% in the context of a normally functioning endothelium.

Functional Alterations

As well as alterations in vascular structure, there are also alterations in VSMC (endothelium-independent) function associated with CVD risk. Although the mechanisms for developing endothelial-independent dysfunction aren't fully understood, The impact of an increased ROS on VSMC's has been highlighted. This increased ROS was associated with hypertension, hyperglycemia, hypercholesterolemia and CVD have been postulated as a source (14, 236). The

increased ROS inhibited NO production thereby inhibiting VSMC function (endothelium-independent mechanism). This also inhibited soluble guanyl cyclase (sGC) and cyclic guanosine monophosphate (cGMP)-dependent kinases thereby inhibiting VSMC relaxation and consequently vasodilation in the context of a normally functioning endothelium (236). Similarly, increased ROS attenuated the biotransformation of nitroglycerin by inhibiting mitochondrial aldehyde dehydrogenase (ALDH) activity thereby further reducing NO uptake. The net result is an impaired endothelium-independent (VSMC-mediated) vasodilation in the context of a normally functioning endothelium. This would result in an abnormal truncated CAR% response with a healthy, functioning endothelium. Endothelium-independent dysfunction has been shown to be associated with cardiovascular risk, and independently predictive of outcomes in cardiovascular disease patients (42, 235, 249). This demonstrates the importance of endothelium-independent factors affecting carotid artery vasomotion. This further demonstrates the importance of endothelium-independent mechanisms on vascular health. Maruhashi et al (2018) suggests that endothelial function cannot be accurately measured from vasomotion alone (236). They suggest that endothelial function has to be interpreted in the context of endothelium-independent nitroglycerin-induced dilation (NID). They further suggest that, if there are both impaired endothelium-dependent and endothelium-independent vasomotion. It may be impossible to discern whether the impaired vasodilatory response is as a result of endothelial dysfunction alone or concurrent abnormalities in both. This highlights the importance of measuring NID in CAR%. The impact of sublingual glycerol trinitrate (GTN – a NO donor) on carotid artery reactivity has previously been investigated in healthy participants. Thijssen et al (2011) found that GTN caused both carotid artery and femoral artery dilation in

both old and young participants (250). They demonstrate the safety and feasibility of using GTN to investigate carotid artery NID. Similarly, GTN administration has been demonstrated to be safe in both patients with CVD and carotid artery disease (251). This was not feasible within this study as the research was undertaken by a non-clinician. This therefore highlights the key role endothelium-independent dilation plays in driving vasomotion and also highlights the safety and efficacy of using sublingual GTN to measure carotid artery NID. However, our group have demonstrated previously that CAR% is correlated with CVD risk and predicts AEs in PAD patients without measuring NID (54, 111).

Taken together, the impact of endothelium-independent vasodilation highlights a key future area for research. This requires further research investigating the impact both carotid artery endothelium-dependent and -independent mechanisms have on risk in CABG surgery. In such a study, participants would first undergo the CPT to investigate endothelium-dependent vasomotion. They would then undergo carotid artery diameter testing alongside sublingual GTN with a sufficient wash-out period after the CPT. The combination would allow researchers to investigate the impact of endothelium-dependent, and -independent dysfunction on patient outcomes. It may be that mild dysfunction in either endothelial-dependent or -independent mechanisms are compensated for via the subsequent mechanism. The presence of two inter-related mechanisms affecting carotid artery dilation may also explain the variance in response seen in this study as well as others. This variance may be due to the degree of (dys)function as well as the presence or absence of compensatory mechanisms.

7.5. Future Work (Scan Acquisition and Analysis)

A further area for potential development is advancement in scan acquisition and analysis. During the process of undertaking this PhD, another group have published extensively on the utilisation of 3D tomographic ultrasound in imaging vessels in 3D (252-254). This is a technology that may be used to gain a more complete measure of carotid artery vasomotion. This also offers an opportunity to undertake a more complex analysis of vasomotion using response curve analysis to better capture complex vasomotor responses.

Tomographic 3D Ultrasound

When the carotid artery is investigated using ultrasound, a segment of artery is imaged along a single 2D plane. The carotid artery response is estimated by measuring the distance between proximal and distal walls of the artery along a single plane (***Figure 16***). This is used as a surrogate measure of arterial diameter. However, this is associated with two key limitations. Vasomotion is a complex response associated with circumferential squeezing/relaxation throughout the diameter of the vessel. This results in a change in vessel lumen volume. Whereas, current 2D techniques measure the distance between arterial walls. The uniformity of vasomotion and the resultant change in arterial diameter in response to the CPT has not been investigated. A loss of mechanical stability which resulted in non-uniform vasomotion has been demonstrated previously in bovine carotid arteries and has been measured using computational modelling (255, 256). This is termed “arterial buckling” and has been demonstrated in response to hypertensive pressure, reduced axial stretch or changes in wall stiffness and dimension (255, 256). The contraction of arteries resulting in tortuous phenotypes associated with age and CVD risk are also associated with non-uniform vasomotion (255, 257). This is represented in ***Figure* 16**

below. It is not known to what extent carotid arteries suffer arterial buckling or non-uniform vasomotion throughout the CPT. There is also an increased risk of buckling in tortuous arteries (257). It may therefore be more accurate to measure change in lumen volume rather than wall distance as a measure of vasomotion.

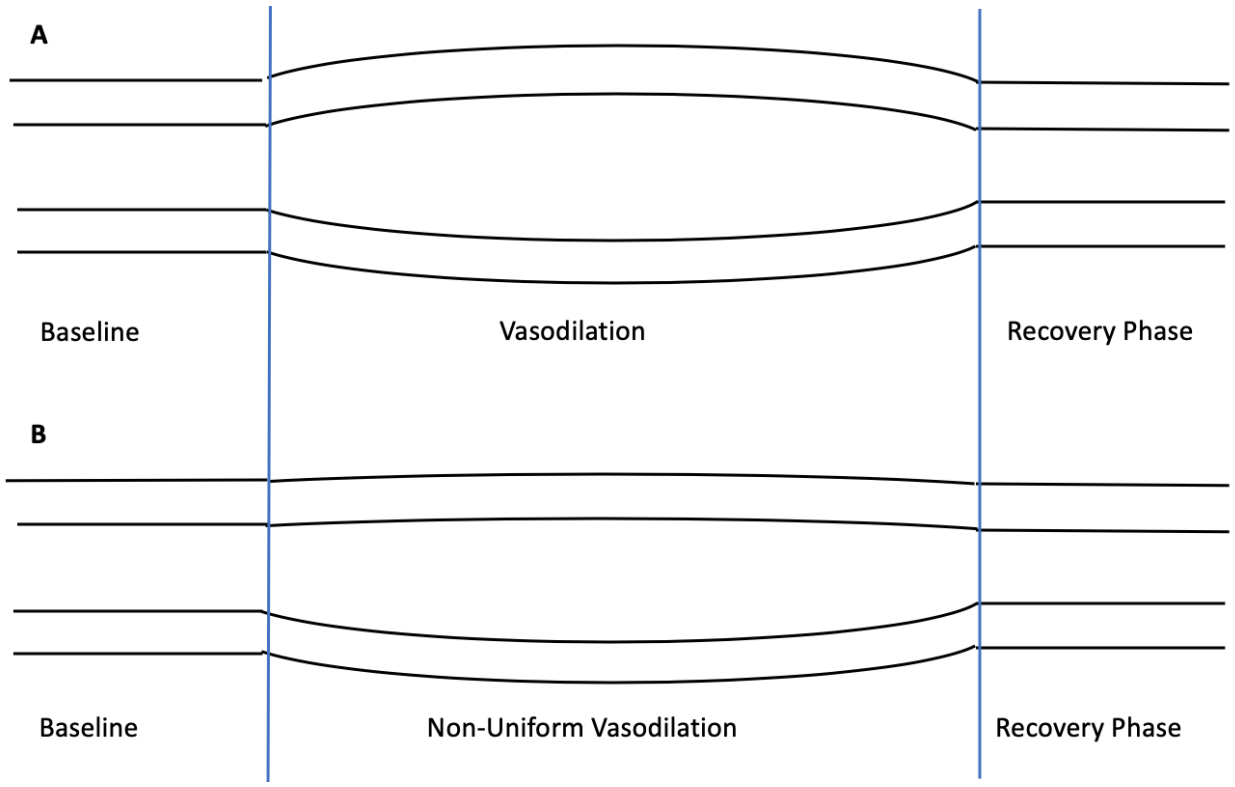


***Figure 16*)** A diagrammatic representation of the vascular response throughout the three phases of the CPT. Including the "baseline", "vasodilation" phase through SNS stimulation and the "recovery" phase. **A**) A normal dilation phase in which both proximal and distal walls dilate equally throughout the vasodilation phase and **B**) A non-uniform dilation phase in which the distal wall moves to a greater extent than the proximal wall.

As arteries are 3D structures, it is difficult to capture an in-focus artery along the entire segment of vessel along a single plane. This is particularly difficult as arterial tortuosity increases with age

and CVD risk factors such as hypertension and diabetes mellitus (***Figure 17)*** (258). This therefore forces practitioners to concentrate on a limited section of in-focus artery further limiting the size of segment analysed throughout the CPT. The use of 3D tomographic ultrasound may offer a relatively simple method to produce 3D reconstructed maps of vasomotion. In 3D tomographic ultrasound, a specifically designed ultrasound probe is used to take a series of transverse images throughout a segment of the vessel. These images are then used alongside software to create a geometric mesh (253). This can be used to create computational models of vessel volume. Feurer et al (2012) demonstrated that tomographic ultrasound is an accurate and reliable method to measure both carotid artery volume and plaque structure. They also demonstrated its potential use in identifying high-risk plaque (210). Similarly, Ball et al (2018) recently demonstrated that 3D tomographic ultrasound can be used to calculate both vessel and plaque volume in patients with both symptomatic and asymptomatic carotid artery stenosis (252). The same group have also validated the use of contrast-enhanced 3D tomographic ultrasound in patients with PAD (254). They further report that tomographic ultrasound performed better than B-Mode ultrasound, CT and MR imaging when calculating volume measurements of an *ex vivo* phantom model of porcine arteries (253). Taken together, this highlights the potential use for 3D tomographic ultrasound to non-invasively measure the volume of carotid arteries in 3D. This may overcome potential limitations of current 2D methodologies including non-uniform vasomotion and an inability to measure a vessel along a single plane. This may also add to the accuracy of ultrasound as a measure of vasomotion. To our knowledge, there are currently no studies that have investigated the use of tomographic ultrasound to measure carotid artery reactivity. This

may be due to the inherent difficulties in capturing dynamic changes through time in a 3D structure.

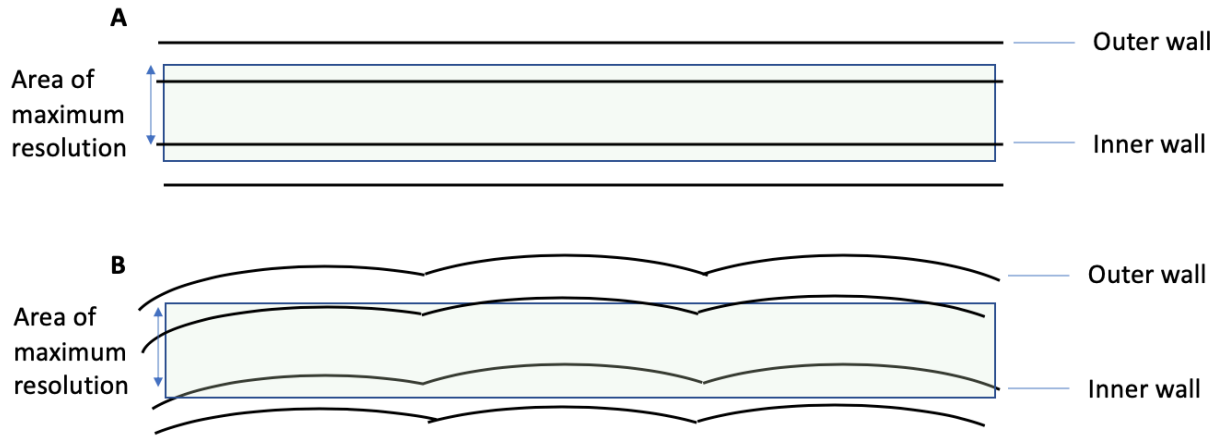


Figure 17) A diagrammatic representation of the process of vascular ultrasound using B-mode 2D ultrasound. **A**) A normal straight artery in which all areas of the inner wall of both the proximal and distal aspects of the artery are within the area of maximum resolution and **B**) A tortuous artery in which the proximal aspect of the inner wall are partially outside the area of maximum resolution producing sub-optimal scan resolution.

Response Curve Analysis

A second limitation may be in image analysis. CAR% is the maximum change in diameter described as a percentage above or below baseline. Although this is a valid measure of change in diameter and has been previously demonstrated to predict risk in PAD patients (54), this may be an over-simplification of the complex response which occurs throughout the CPT. In particular, some participants demonstrated an initial small dilation followed by constriction. The physiological impact of this and the impact on patient outcomes is currently unknown. Similarly,

it is unknown to what extent “delayed” response is a marker of endothelial dysfunction. A delayed response has also been correlated with increased cardiovascular risk (111). This may mean that it takes a larger stimulus (and therefore a relatively longer time spent in the CPT) to reach a threshold to cause dilation due to the reduced expression of pro-dilatory adrenoreceptors. Van Mil et al (2017) demonstrated a significant reduction in peak response (CAR%), total response (CAR_{auc}) and diameter at 90 seconds (CAR_{90}) in young (24±3) vs old participants (61±8). They also found a reduction in CAR% (P: 0.060), CAR_{AUC} (P:0.034) and CAR_{90} (P:0.037) in participants with the presence of CVD risk factors compared to healthy individuals (111). Similarly, Buckley et al (2018) found CAR% and CAR_{AUC} were significantly correlated to CVD risk factors (229). Taken together, these studies demonstrated that peak response, diameter response and timing are significantly delayed in older participants and those at an increased CVD risk (111, 229). This highlights key characteristics associated with the CPT response, which were not captured when calculating peak CAR% alone. As the diameter was recorded continuously throughout the CPT, there may be an opportunity to perform response curve analysis. This may allow the creation of a set of healthy reference values incorporating known differences in physiological response to the CPT associated with both gender (221, 259) and age (260). A goodness-of-fit analysis can then be investigated to analyse divergence from healthy references values. This can be used to identify patients with an abnormal vasomotion response to the CPT and may capture abnormal response characteristics such as “mixed” initial dilation followed by a constriction or delayed response time currently not captured using CAR%. These response characteristics have been associated with CVD risk and may also be additional markers of dysfunction.

7.6. Future Work (Practical Considerations)

The current techniques used to investigate CAR% were associated with two practical considerations or areas for potential development.

A key practical consideration is the use of hand-held probes in ultrasound. Free-hand duplex ultrasound is current clinical practice used in real-time to image and characterize (patho)physiology (261, 262). This is highly dependent upon the practitioner and does not require standardization for reproducibility. As this is not used to measure a dynamic response, this is not as dependent upon a standardized static baseline. In the CPT, as participants moved to place their hand in water, one of the risks was movement of the probe. This would result in a shift from baseline or artefacts in the trace which were subsequently removed prior to analysis (54, 130). To avoid this, physiological landmarks were identified prior to recording and used as reference points (49). The use of mechanical probe-holding devices has been investigated in the context of Flow Mediated Dilation (49). The use of probe-holding devices has been associated with an increased FMD reproducibility (263). However, another group demonstrated that probe-holding devices did not improve reproducibility when scanning was performed by experienced practitioners who adhere to strict guidelines (264). Although these guidelines were developed for FMD, they can be broadly applied to CAR%. However, there are no current expert consensus guidelines for CAR%. Taken together, standardization and an ability to avoid artefacts and movement whilst scanning is key. This is particularly important when trying to quantify vasomotion. To our knowledge there are currently no publications addressing the reliability or reproducibility of CAR% in clinical groups. Importantly, CAR% has been demonstrated to have

good same-day and next-day reproducibility in healthy participants (115). There are also currently no consensus guidelines on the use of CAR% or accepted requirements to be considered proficient. The use of standardized guidelines along-side mechanical probe-holding devices may reduce the considerable time taken to become proficient and may also improve reliability in already proficient researchers. This may be a key step in translating CAR% from the research laboratory into clinical practice.

A further practical consideration relates to the time-consuming nature of analysis. If a measure of endothelial function is going to be used in clinical practice. It is key that it can be used as a real-time read-out of endothelial function. The results should also be produced quickly and simply to aid real-time clinical decision making. When calculating CAR%, all analysis was performed offline. A ROI was manually drawn, and automated wall tracking software was used to calculate diameter. Ten second intervals were manually drawn for the entire CPT and the average diameter calculated for each ten second bin. This was then input in to a pre-coded excel spreadsheet and CAR% is calculated (14, 54, 130). In 3D ultrasound, segmentation algorithms are used, and slice volume is then calculated slice-by-slice. This is again time-consuming and would also require additional manual statistical analysis. If these techniques are going to be implemented into clinical practice, this requires significant automation. Image analysis has vastly benefitted from automated wall-tracking software (49). However, the further automation of identifying a ROI in 2D analysis and vessel segmentation in 3D analysis is key. The improved resolution of current-generation and next-generation ultrasound machines mean greater contrast in vessel wall (lines of Pignoli) vs surrounding tissue. This along-side improvements in wall-tracking software means improved axial resolution and distinct arterial wall borders. This

results in a greater potential for automated generation of an ROI or vessel segmentation algorithms (49). The use of artificial intelligence may be able to "train" algorithms to automatically select an ROI or segment a vessel, accounting for variance in angle of insonation/vessel and then calculate vasomotion dynamically throughout the CPT. A key limitation in current automated analysis and segmentation is in the presence of scanning artefacts. Physiological artefacts such as calcification and increased IMT/atheroma pose a considerable problem and lead to incorrect analysis (265). This along-side procedural artefacts such as movement throughout the CPT, participant swallowing, movement during breathing or arterial pulsatility remain a barrier to automation. It may be possible to "train" artificial intelligence algorithms to manage or avoid these artefacts alongside the use of electrocardiogram-gated (ECG) analysis to minimize the impact of carotid artery pulsatility (49).

The subsequent automation of statistical analysis is relatively simple, requiring an automated work-flow including macros which deposit the raw data in to a pre-coded spreadsheet. This can then automatically calculate variables such as peak response. Similarly, pre-coded spreadsheets have been developed for automated FMD analysis (266). Taken together, the potential improvements in 3D scan acquisition and analysis may be of clinical benefit. However, the complex and user-dependent nature of sonography along-side the laborious nature of image preparation and analysis make this unlikely to be integrated into clinical practice currently. The development of specialist guidelines along-side the use of mechanical probe-holding devices may reduce the significant training required. The development of automated analysis workflows and algorithms will further reduce the time-burden and are required before this tool can be used more widely in a research setting and in clinical practice.

A key consideration when implementing any technology in to clinical practice is the cost of this technology. The cost is associated with the initial cost of equipment and subsequent upkeep, the time in which the test takes and the level of expertise required to undertake the test. In this study, the initial set-up is relatively inexpensive. The most expensive part is the ultrasound machine used. Although each unit is expensive, ultrasound machines are common within hospitals. Although the initial scan is not time-consuming and only takes approximately four minutes, the post-recording processing and analysis is time-consuming. It is therefore key that this is expedited as outlined above. It is also time-consuming to develop the relevant expertise in carotid artery ultrasound. These scans are typically undertaken by highly trained clinical practitioners. The use of probe-holding devices may again reduce the training time required to become proficient. This will again further reduce the cost associated with utilizing carotid artery ultrasound clinically.

7.7. Thesis Conclusions

Study Conclusions

The over-arching aim of this thesis was to investigate the use of the CAR% to non-invasively measure endothelial function and to investigate if this predicts risk in patients undergoing CABG surgery. This thesis was separated in to two distinct but inter-linked studies. First a mechanistic study which investigated the mechanistic regulation of CAR%. Second, a clinical study which investigated the predictive capacity of CAR% in CABG patients.

In study one, we demonstrated that CAR% is not significantly driven by the increase in BP throughout the cold pressor test. This study, together with the literature suggests that CAR% is

predominantly driven by neuronal mechanisms. This adds weight to the validity of CAR% as a surrogate measure of endothelial function and therefore vascular health. In study two, the impact of CAR% was investigated in coronary artery disease patients. There was a weak inverse correlation between CAR% and EuroSCORE II (p: 0.0114 r: -2.85). This suggested that participants at the highest surgical risk (EuroSCORE II) also had the poorest endothelial function. This adds some weight to the validity of using CAR% as a measure of systemic vascular health. EuroSCORE II alone did not predict AEs in this group (p: 0.60). CAR% alone did not predict AEs in this group (p:0.51). This may be as a result of the low incidence of AE's in this study. When combined, CAR% marginally improved the ROCauc in multivariable analysis. This may be suggestive of an improvement. Although this was still not predictive (r:0.60 vs 0.615). Although this study is unable to conclusively investigate if CAR% predicts risk in CABG patients. To our knowledge, this is the first study investigating the ability of CAR% to predict risk either alone or combined with EuroSCORE II in central arterial disease patients.

This thesis, together with the literature adds further validity to the use of CAR% as a research tool. It may add some evidence to the usefulness of CAR% as a tool to predict risk when combined with EuroSCORE II. Although results remain inclusive. It further highlights a number of key areas which may further improve the validity and accuracy of CAR% as a measure of endothelial function. These improvements together with a larger sample size and therefore higher incident number would be required to conclusively investigate if CAR% improves the predictive ability of EuroSCORE II.

Clinical Implications

Endothelial function has been demonstrated to be independently predictive of risk in cardiovascular disease groups. EuroSCORE II performs more poorly in the highest, and lowest risk patients. This makes clinical decision making in these group more difficult. We provided some evidence that CAR% may offer a simple method to measure endothelial function as a surrogate of vascular health. We also provide some limited evidence that CAR% may improve the predictive capacity of EuroSCORE II, although this was inconclusive. The identification of endothelial dysfunction and therefore poor vascular health may help clinical decision making in "borderline" patients who are candidates for both CABG surgery and conservative medical treatments as well as those at the highest surgical risk. Importantly, the identification of endothelial dysfunction could identify a potential target for treatment. This would allow endothelial dysfunction to be improved prior to surgery thereby reducing their risk.

The ability to accurately predict risk is key in clinical decision making as well as in providing patients with the most accurate information possible to allow them to make an informed choice about their care. The risk of any surgical procedure is dependent upon a large number of both modifiable and non-modifiable factors. It is therefore key to approach risk prediction holistically rather than as a "one size fits all" approach. It is important to encompass the largest number of factors possible which are known to impact outcomes within a specified clinical group. The EUROSCORE II tool encompasses a large number of factors and allows clinicians to offer a more personalised approach to risk prediction. In line with part 2 of the PROGRESS framework, EuroSCORE II encompasses a large dataset and can be modified to encapsulate differences in demographics such as age and gender. It is also easily calculable and importantly can be readily updated. These factors facilitate the continued usefulness of EuroSCORE II, particularly the ability

to update these tools to include new and emerging predictive factors. If CAR% is going to be included in future updated risk prediction tools it is key that, this is easily incorporated in to current and future risk predict tools, that it is derived from large and diverse datasets, that it can be easily and reliably performed in a clinical setting and that the results are robust. As with all risk stratification tools, it is also key that there remains an ability to further update and modify this tool as information emerges. CAR% has previously been demonstrated to be reliable. The process of capturing and analysing CAR% can be expedited as described in the "future work" section of this chapter. The process of undertaking and analysing CAR% is easily modifiable and therefore can be easily updated as more information is garnered and improvements are made to both the workflow and analysis of CAR%. This makes CAR% relatively simplistic to incorporate in to clinical practice.

Incorporating endothelial function into clinical practice

As discussed above, measures of endothelial function have a potential benefit in risk prediction in CABG surgery. We provided some evidence that CAR% may marginally improve the predictive capacity of EuroSCORE II. Although this was not definitive and therefore further studies including larger studies involving more participants and therefore AEs. If future studies further demonstrate the usefulness of CAR%, it may be implemented into clinical practice. This is reliant upon the development of specific guidelines to standardize testing alongside the implementation of probe-holding tools to reduce training time and improve inter-user reliability in less experienced practitioners. This should be developed along-side tools and workflows that automate and expedite image processing and analysis. The standardization of image acquisition

along-side automated analysis would allow the creation of a large database of reference values. This would include healthy individuals incorporating differences in endothelial function associated with age and sex. The creation of a set of standardized healthy reference values would allow practitioners to compare an individual's response to these reference values using goodness-of-fit analysis to identify endothelial dysfunction. The presence or absence of endothelial dysfunction can be used as a binary measure that can be incorporated into current risk stratification tools. The current risk stratification tools include variables that are known to affect endothelial function. However, they do not measure endothelial function. They simply note the presence of factors which negatively affect endothelial function and use that to predict risk. They do not include atheroprotective factors, such as exercise, or measure the net impact, of both atheroprotective and atheroprone factors have on endothelial function. This may be a significant flaw. The carotid artery response may be uniquely placed to offer a simple and non-invasive method to measure and quantify the accumulative effect of both atheroprotective and atheroprone risk factors on central artery vascular health. This allows a move away from epidemiological risk scores derived from large datasets to a personalized approach to risk stratification at the point-of-care.

The CAR% test may also highlight a key potential therapeutic target to optimize at-risk patients prior to surgery. Endothelial-dependent coronary artery vasodilation has been improved using a variety of pharmacological interventions including angiotensin-converting-enzyme inhibitors, β-hydroxymethylglutaryl-coenzyme A reductase inhibitors and antioxidants (267-270). Importantly Buckley et al (2019) recently demonstrated a 12-week exercise intervention reversed endothelial dysfunction and restored endothelial function in participants at an increased risk of

cardiovascular disease measured using CAR% (229). Similarly, Hambrecht et al (2000) also demonstrated that a 4-week intensive exercise intervention reversed coronary artery endothelial dysfunction in patients with coronary atherosclerosis (271). They measured coronary artery endothelial function using quantitative angiography in response to acetylcholine. Taken together, this highlights two key benefits of measuring CAR%. First, identifying patients who have endothelial dysfunction and who are therefore at an increased risk. Second, undertaking both pharmacological and non-pharmacological lifestyle interventions in these groups. Thereby improving endothelial dysfunction or reversing dysfunction prior to surgery. This includes reducing modifiable risk factors and undertaking exercise-interventions to reverse endothelial dysfunction. These interventions in the form of "pre-habilitation" may be used to improve endothelial function or reverse endothelial dysfunction and therefore reduce risk prior to surgery. A growing body of research demonstrates the benefits of "pre-habilitation" to optimize patients prior to cardiac surgery (272). These interventions have improved intensive care unit (ICU) discharge times, shortened length of stay, improved cardiovascular fitness, and reduced complications in CABG patients (272). There is currently no consensus on optimum intervention types. It is not known to what degree these benefits of "pre-hab" are as a result of improved exercise tolerance or improvements in endothelial function. It is possible these benefits are as a result of improved endothelial function/reversal of endothelial dysfunction. To our knowledge, there are no studies investigating the degree to which the known benefits of "pre-hab" are as a result of improved endothelial function alongside classical exercise benefits such as improved cardiovascular fitness. The simplicity and non-invasive nature of carotid artery reactivity would allow serial measurements to be taken throughout an intervention at the point-of-care, such as

in the community or GP practice. The development of strict guidelines alongside automation would allow non-experts to undertake testing and produce an immediate read-out of endothelial function. This would allow clinicians to monitor the improvement in endothelial function in response to an intervention and adapt it appropriately. Thereby offering a personalized approach to risk assessment and patient optimization prior to surgery. It is hoped that pre-surgical optimization including improving endothelial function in patients with endothelial dysfunction would then translate in to improved outcomes and lower incidences of adverse events in patients undergoing CABG surgery.

Appendices

7.8. Appendix 1 – Search Strategy

A comprehensive and methodological search strategy is key to an effective and accurate literature review. I therefore employed a PICO framework.

Population	Cardiovascular disease
Intervention/Indicator	Endothelial Function
Comparison	Measures of Endothelial Function
Outcome	Vascular Health OR Cardiovascular Disease Outcomes

***Table* 21**) A PICO table describing the search framework used as part of the search strategy.

I first began by trying to understand the current literature of risk stratification/prediction tools on cardiovascular disease. I therefore combined "Cardiovascular Disease", "Cardiovascular Event", "AE", and "Death" together with "endothelial function" and "Endothelial dysfunction". This highlighted a broad range of methods employed within the literature to measure risk in cardiovascular disease. I then differentiated methodologies by "invasive" and "non-invasive" techniques used to measure endothelial function. This was in both the context of cardiovascular disease and in healthy populations. I moved more specifically on to non-invasive measures of endothelial function, combining this with "cardiovascular disease". As the carotid artery was used as a surrogate measure of coronary artery endothelial function. I then began to look specifically at measures of endothelial function within the carotid artery, together with coronary arteries. As this study uses the Cold Pressor Test (CPT) as a SNS stimulus, I looked specifically at measures of

endothelial function in response to the cold pressor test. This is a relatively small field, with a small number of very active researchers. I therefore began to use citation tracking to gain a better understanding of the field. Although the use of the CPT in endothelial function and risk prediction is a relatively small field. The CPT had been used extensively in physiology studies investigating systemic and vascular responses to the CPT.

Critical Literature Review

I performed a comprehensive critical review of the literature. The primary sources were Medline Plus, Pubmed via the National Centre for Biotechnology Information (NCBI). This identified 526 studies related to the research area, of which 87 were relevant to my research. I accepted 64 studies, of which 17 were methodological, 30 investigated endothelial function, 5 epidemiological and 15 risk prediction. Of the 30 studies investigating endothelial function, the most important papers are firstly (33) describing endothelial function. The seminal paper by Dunker et al first described a potential mechanism by which endothelial function is lost in atherosclerosis and cardiovascular disease using specific α- and β-adrenoreceptor inhibition (273). The importance of endothelial function has been demonstrated in a number of pre-diseased and diseased states including in patients with moderate and advanced cardiovascular disease. Throughout the literature review, I found that there were a number of techniques that had been used to investigate endothelial function. Within the literature, there are 17 articles directly or indirectly investigating endothelial function as a method to predict risk in cardiac populations. These can be broadly divided in to “direct” methods which investigate endothelial function within the coronary arteries and “indirect” which measure a surrogate of coronary

artery endothelial function. Schachinger et al first investigated the prognostic ability of endothelial function (42). They directly investigated coronary artery endothelial function using intracoronary infusion of Ach and the cold pressor test alongside coronary angiography. Importantly Hays et al confirmed this (53). Due to the expensive and invasive nature of direct coronary artery endothelial function measurement, indirect methods were subsequently developed. The most completely researched method of indirectly measuring coronary artery endothelial function is using Flow Mediated Dilatation (FMD) (64). Although FMD has been researched extensively, there are significant limitations when using peripheral arteries as surrogate measures of central artery endothelial function. Experiments within our group demonstrated that the carotid artery endothelial function correlates with coronary artery endothelial function. It was also demonstrated by others that the carotid artery is more similar to the coronary artery in both structure and function. Most importantly, others within our group demonstrated that the carotid artery response (CAR) to the cold pressor test measured non-invasively using carotid artery ultrasound predicts risk in patients with PAD (54). We therefore hypothesized that carotid artery response to the cold pressor test and measured non-invasively using carotid artery ultrasound predicts risk in patients with significant coronary artery disease undergoing coronary artery bypass graft surgery.

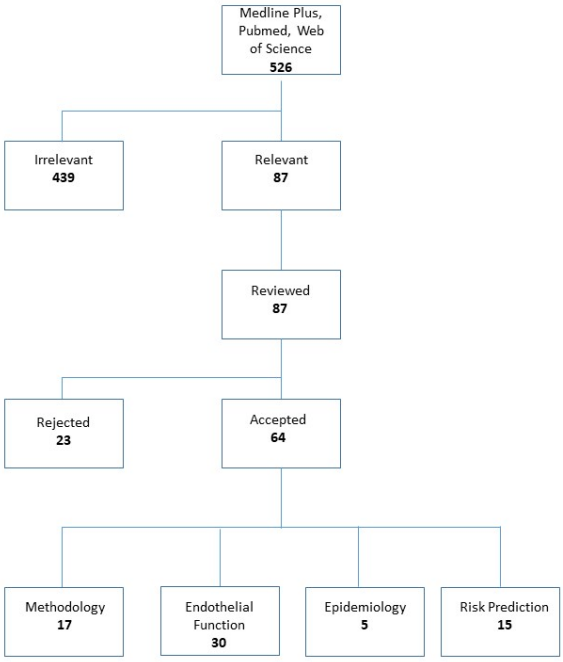


Figure 18)) A Prisma diagram of the available literature on coronary artery endothelial function and endothelial function in risk prediction.

7.9. Appendix 2 – Study 2 Scatterplots

The scatterplots below are produced during the analysis of study 2. These can be used to visually inspect the spread of the data.

This panel demonstrates the whole-group demographic data of participants included in study 2.

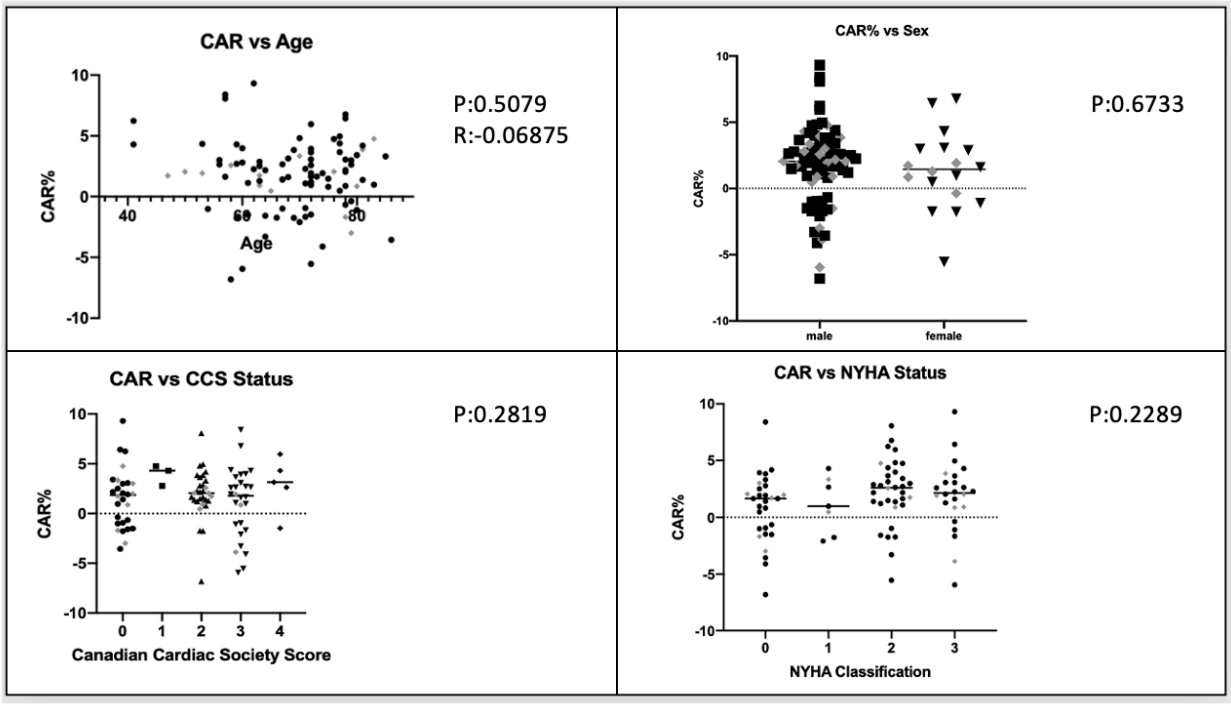


***Figure* 19**) Scatterplots of Age, Sex, CCS score and NYHA score. Participants in grey had post-operative AEs.

The relationship between CAR% and BMI was then investigated. This was investigated both as a continuous variable and when participants are grouped according to NHS guidelines for BMI.

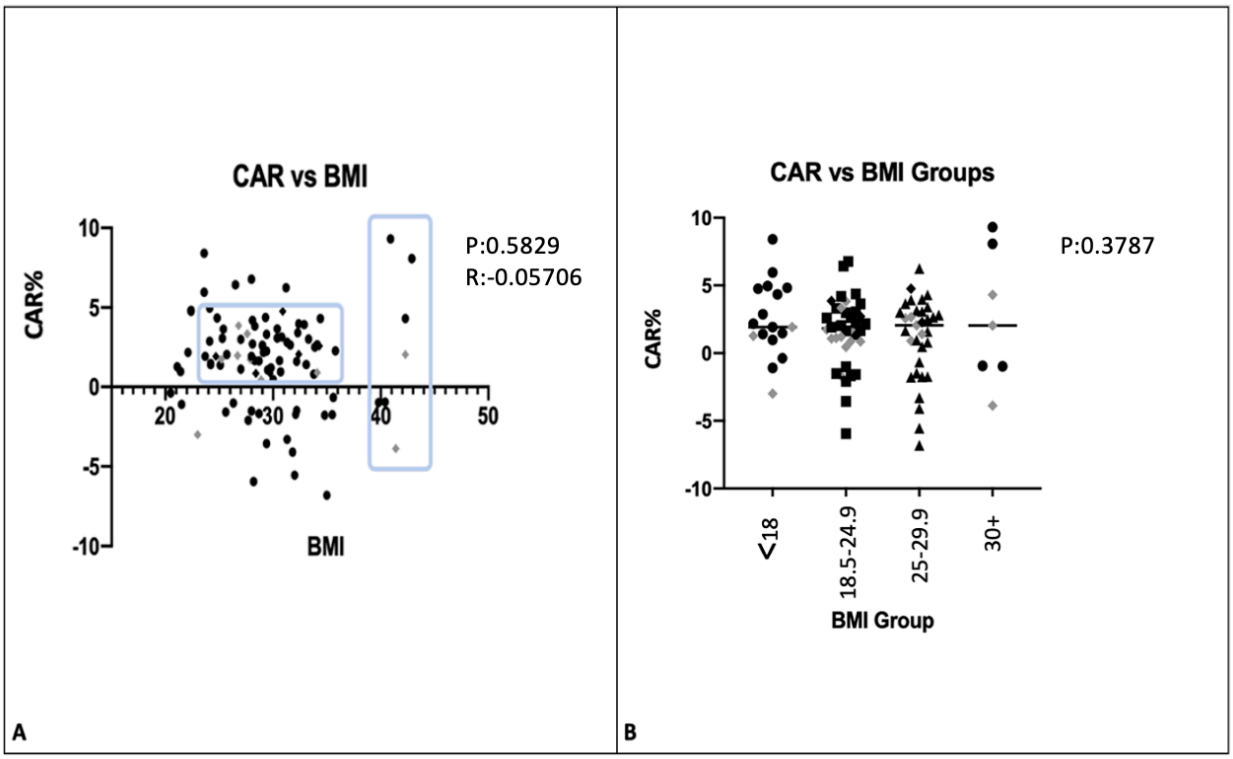


***Figure* 20**) Scatterplots of CAR% vs BMI. A) A correlation of CAR% vs BMI in this group. There appears to be a number of "clusters". B) CAR% in participants when grouped according to their BMI group according to NHS guidelines for BMI. Grey datapoints are those who had AEs. Black datapoints had no AEs.

The relationship between CAR% and CVD risk factors was then investigated.

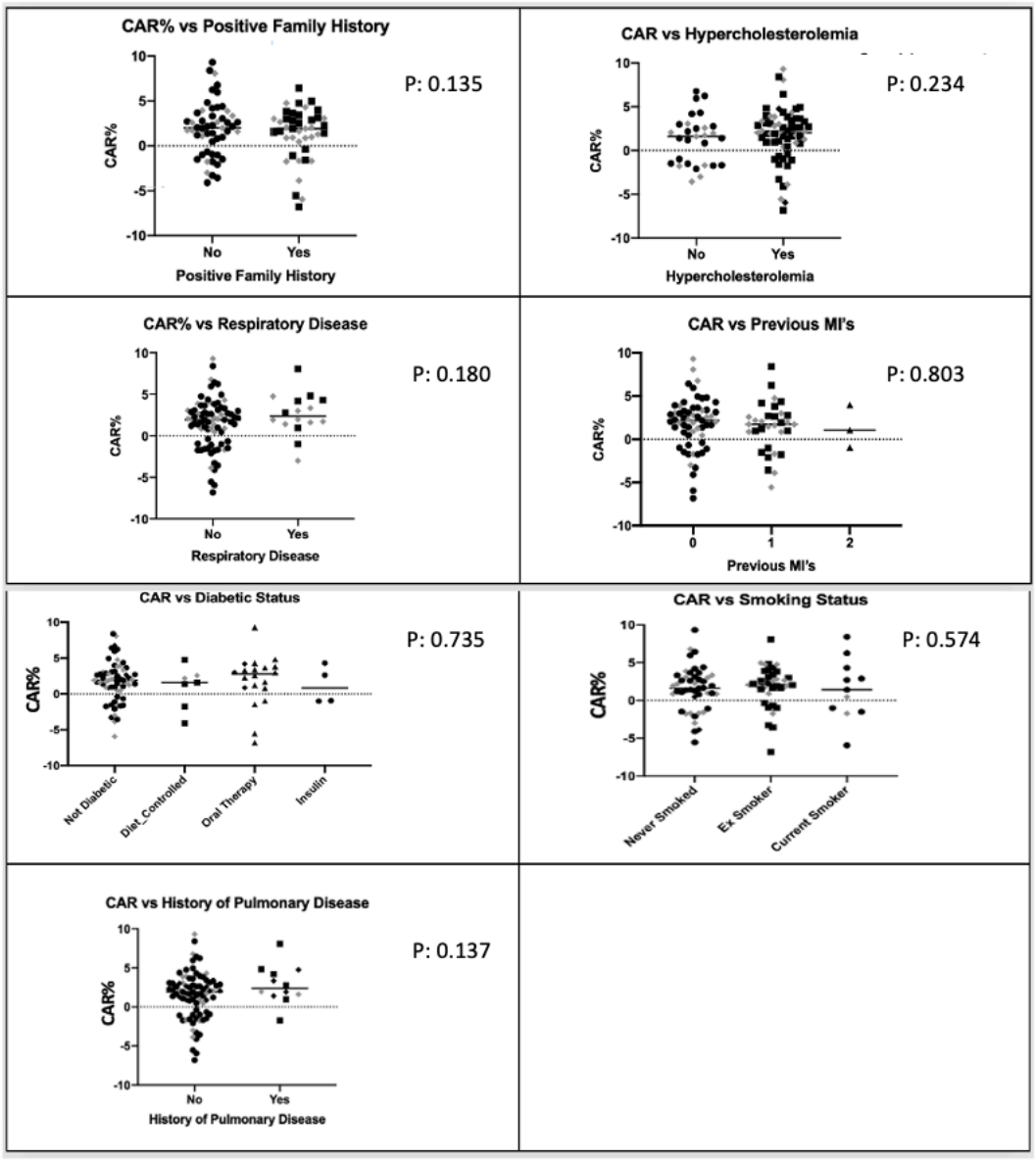


***Figure* 21)** Scatterplots of Family History and Risk factors for developing cardiovascular disease and AEs in CABG surgery. Grey datapoints are those who had AEs. Black datapoints had no AEs.

The relationship between CAR% and markers of disease severity was then investigated.

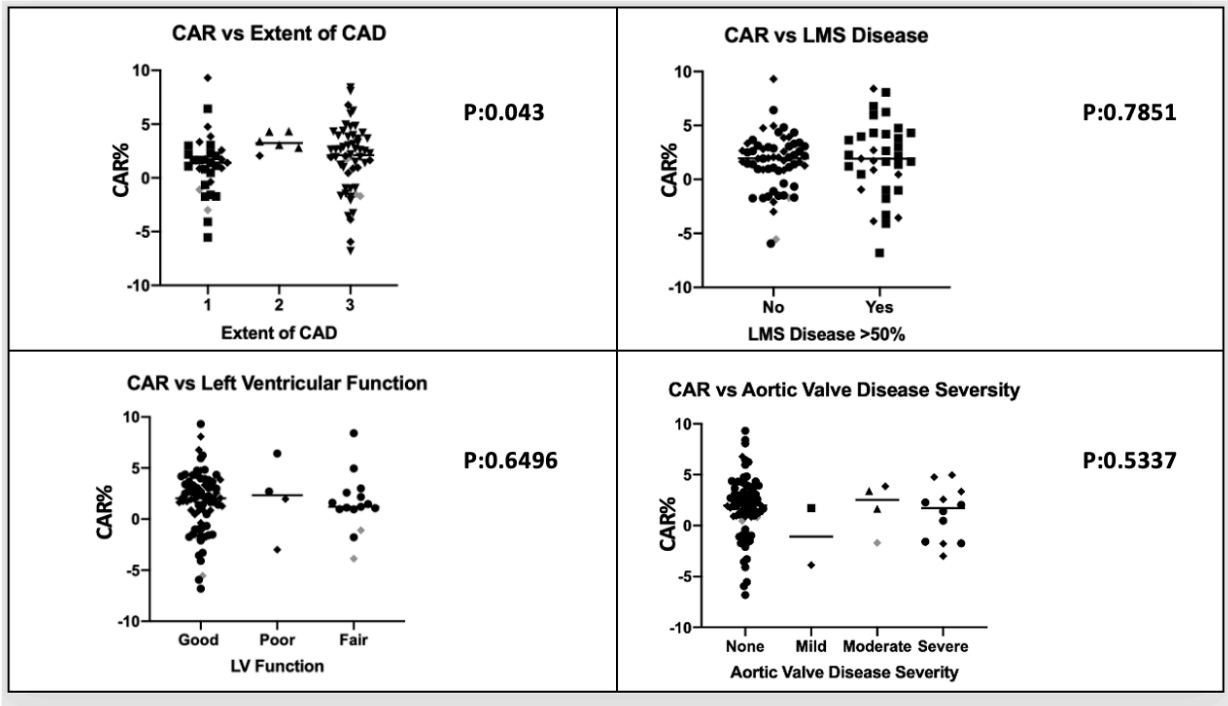


***Figure* 22)** Scatterplots of markers of disease severity. Grey datapoints are those who had AEs. Black datapoints had no AEs.

8. Chapter 8: References

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