Examining endothelial function in humans in vivo:

improving guidelines and exploring novel measures

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from Radboud University Nijmegen on the authority of
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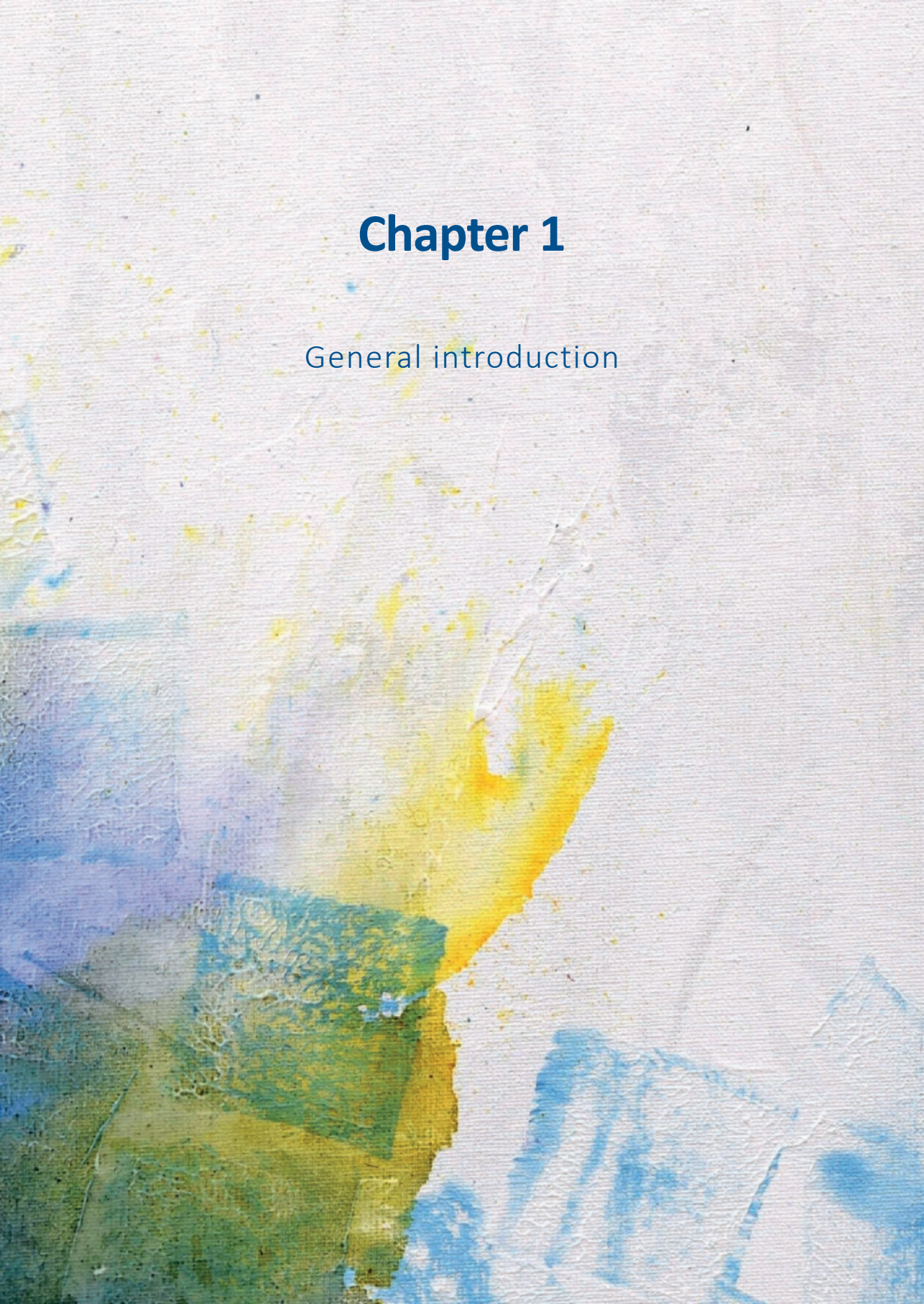
Chapter 1

General introduction



The cardiovascular system

The circulation of blood was one of the landmark discoveries in medical history. Sir William Harvey (1578-1657) might be considered the founder of modern day cardiovascular physiology. He was the first to describe the circulation of blood following the contracting motion of the heart in the *Exercitatio anatomica de motu cordis et sanguinis in animalibus* ('Concerning the motion of the heart and blood', commonly known as De Motu Cordis).[1] Interestingly, Harvey's ideas were introduced in a scientific era dominated by the church, where it was 'believed' for over 1,400 years that blood was produced in the liver and heart, being consumed in the muscles. His research and experiments laid the foundation of modern day knowledge on the cardiovascular system. The cardiovascular system consists of the heart and the blood vessels, and is responsible for the distribution of nutrients and oxygen throughout the body. During normal circulation, the heart contracts, resulting in the emission of blood out of the heart into the arterial blood vessels. After delivering nutrients and oxygen to the periphery, the deoxygenated blood, which now contains waste products and CO_2, is transferred back to the heart, where it is re-oxygenated in the pulmonary circulation, before repeating its original circulation.

Atherosclerosis and the endothelium

Cardiovascular disease and morbidity are responsible for 45% of all deaths annually (more than 4 million people), being the leading cause of death in Europe.[2] The principal contributor to the progression of cardiovascular disease is the process of atherosclerosis.[3] It is of key importance to understand the development of atherosclerosis, which may be realised through exploring the artery wall itself. The wall of the artery consists of multiple layers; the intima (inner), media, and outer adventitial layer. Atherosclerosis represents the pathologic change, initially affecting the intima of arteries, that is characterized by the focal thickening of the sub-intimal space, often due to lipid accumulation.[4] More specifically, when the arterial wall is damaged or dysfunctional, an inflammatory response occurs.[3] Through continuous exposure to potentially detrimental stimuli, this response can become excessive, resulting in abnormal accumulation of white blood cells (leukocytes, foam-cells and macrophages), cholesterol and vascular smooth muscle cells in the sub-intima.[5, 6] This subsequently can ultimately lead to pathologic narrowing and inadequate blood supply to the distal tissue. The earliest atherosclerotic lesions are commonly seen in the intima of major vessels in childhood or adolescence.[7] Several known risk factors contribute to the development of accelerated atherosclerosis, such as hypertension, hypercholesterolemia, diabetes, smoking, and inactivity.[5, 6, 8-18]

Endothelial function and dysfunction

The inner layer of the vascular wall is lined with endothelial cells. Whilst the endothelium plays several physiological roles, maintenance of the adhesive properties (i.e. platelet activation and inhibition) and vascular tone (i.e. by maintenance of blood flow) are of most clinical interest.[5, 6, 19] Furchgott and Zawadzki demonstrated that endothelial cells were responsible for the production of an endothelium-derived relaxing factor (EDRF).[20] This Nobel prize winning research eventually resulted in the identification of endothelium-derived nitric oxide (NO),[21] an important contributor to several physiological processes, including maintenance of the vascular tone.[20, 22, 23] The release of NO depends on a variety of stimuli, including increased blood flow and consequent shear stress, which transduces vasodilation mediated by vascular smooth muscle cell relaxation.[23-26] When the endothelium dysfunctions, the vascular tone and adhesive properties can be disrupted. This may predispose arteries to enhanced responses to vasoconstrictor substances, platelet aggregation to the vascular wall and, eventually, the process of plaque formation.[22, 23] Endothelial dysfunction is an early manifestation of atherosclerosis, which precedes structural signs of vascular alteration.[3, 6, 27] This makes the endothelial dysfunction an important target for early intervention in efforts focussed on CVD prevention.[14]

Whilst the process of atherosclerosis occurs throughout the body, the coronary circulation is particularly susceptible to the development of endothelium dysfunction. Studies have shown that intracoronary infusion of acetylcholine, a potent endothelium-dependent vasodilator, can induce paradoxical vasoconstriction in the coronary arteries, implying the presence of endothelial dysfunction.[28-31] Such constriction occurs in patients with established CVD, but also in individuals with angiographic 'normal' coronary arteries who may be at high risk of future atherogenic development. Indeed, coronary artery responses to acetylcholine strongly predict the occurrence of future CV events. These findings highlight the key role of the endothelium in atherogenesis and CVD progression.

Measuring vascular function and health in humans

Developing and validating reliable techniques to measure vascular health with strong, independent, predictive capacity for future cardiovascular disease, represent the Holy Grail.[19] In the past, several tests have been developed and tested. Some of the most commonly used tests will be discussed below (Table 1).

Table 1. Advantages and disadvantages of different vascular tests

Technique	Vascular bed	Advantages	Disadvantages	Stimulus
Coronary angiography	Epicardial conduit arteries	• Direct assessment of coronary vascular bed • Functional and structural assessment • Golden standard	• Invasive • Time consuming • Expensive • Difficulty in performance	Acetylcholine Cold pressor test
Intima-media thickness	Peripheral conduit arteries, often carotid or aorta	• Non-invasive • Surrogacy of coronary structure • Easy to perform • Quick test	• Structural assessment • Dependent on plaque • Added clinical value unknown	
Pulse-wave velocity	Aorta and peripheral arteries (e.g. radial artery)	• Non-invasive • Surrogacy for coronary function (aorta) • Functional assessment	• Limited evidence in periphery • Reliability uncertain	propagation of systolic wave
Flow-mediated dilation	Peripheral conduit arteries, often brachial artery	• Non-invasive • Surrogacy of peripheral arteries for coronaries	• Technically challenging • Large between-study variability • Inconsistency in FMD value	NO-release Shear
Carotid artery reactivity	Carotid artery	• Non-invasive • Surrogacy for coronary function • Easy to perform • Quick test	• Physiology unknown • literature reliant on coronaries	Cold pressor test

Adapted from Flammer *et al*.[32]

Coronary structure. In examining coronary artery disease (CAD), especially in the suspicion of (partial) stenosis, direct assessment of coronary artery structure is the commonly adopted approach. Serendipitously, Mason Sones Jr. was the first to perform coronary catheterization. Whilst attempting to perform cardiac catheterization, but mistakenly inserted the catheter in the right coronary artery.[33] Together with the findings of Drs. Judkins[34] and Amplatz,[35] radiologists who gained access to the left ventricle through percutaneous techniques via the femoral artery, Sones laid the foundation for the current coronary angiography technique. Coronary angiography uses the infusion of contrast dye following coronary catheterization, with simultaneous X-ray pictures to visualise the coronary arteries. This allows for the detection of blockages in the coronary circulation that are the result of lesions and atherosclerotic plaques. This procedure is regarded as the gold standard in assessment of the coronary anatomy, as it accurately defines the extent of coronary luminal obstruction.[36] However, using coronary angiography as a 'screening tool' is not prudent, nor cost-effective in clinical populations.[36, 37] Additionally, coronary angiography is limited in its ability to define the aetiology of the obstruction,[36] and lacks representation of the coronary functionality.

Coronary function. When the invasive coronary angiography technique is combined with infusion of different substances (e.g. acetylcholine, a potent endothelium-dependent vasodilator, or adenosine, nitroglycerin, or papaverine, all endothelium-independent dilators), functionality of the coronary endothelium can be assessed.[38-41] This procedure is commonly regarded as the gold standard technique to examine coronary endothelial function, since it independently predicts acute CV events in patients both with and without CAD, providing strong prognostic value.[38, 41-43] Nonetheless, the potential limitation associated with coronary infusion is the increased risk for complications, involving bleeding, infections, and blood vessel damage. Using coronary infusion on a large scale to assess the risk for CVD is ill-advised, due to its invasiveness, costs, difficulty in performance and it is time consuming.

Peripheral structure (intima-media thickness). Based on the invasive nature of intra-coronary drug infusion and the difficulty of measuring coronary artery function, several studies have explored the potential clinical value of measuring peripheral conduit artery function and structure.[7, 44] Previous studies have shown a good agreement between peripheral and coronary arteries, and, therefore, peripheral arteries have be used as a surrogate for coronary arteries.[25, 44-46]

In 1986, Pignoli was the first to describe the possibility of non-invasively imaging atherosclerotic lesions in peripheral arteries (e.g. the common carotid artery and the aorta) using B-mode imaging.[47] Since then, numerous studies have demonstrated that the common carotid artery intima-media thickness (cIMT) is a strong predictor for future cardiovascular events,[48-50] and that it relates to the development of coronary artery disease.[51-53] Unfortunately, measuring the cIMT relies heavily on the presence of carotid plaques, and according to recent studies,

plaque measurements alone add to CVD risk prediction, and are independent of IMT measurements.[54, 55] Accordingly, it remains unclear to what extent the cIMT measurement is of added clinical value to risk stratification in individuals free from CVD.[56-59] Nonetheless, this easy, quick, non-invasive test remains a popular test in studies on cardiovascular health.

Peripheral structure/function (aortic stiffness and pulse-wave velocity). Other tests to examine peripheral vascular health relate to the stiffening of the arteries. Stiffening of the larger arteries is believed to reflect both structural characteristics of the vessel wall, but also functional aspects of artery health. Stiffness of the cardiovascular system can be assessed locally in an individual artery (i.e. stiffness) or across a vascular bed (i.e. pulse wave velocity). Assessment of stiffness entails measuring the arterial diameter across the cardiac cycle (i.e. difference between systole and diastole), which is related to the pulse pressure, leading to an arterial diameter change for a given increase in (blood) pressure.[60] The aorta is of great interest in determination of regional stiffness, since the aorta makes the largest contribution to the arterial buffering function. The buffering capacity of the aorta is an independent predictor of outcome in a variety of populations, such as hypertensive patients and patients with higher cardiovascular risk.[61-63]

Pulse-wave velocity is the most common and generally accepted measurement of arterial stiffness, a measurement based on the velocity of blood from the heart to a peripheral artery.[61] When the heart contracts, the systolic blood wave propagates through the aorta into the periphery, transitioning from large elastic vessels to the smaller muscular periphery. When the vasculature becomes stiffer, blood will travel faster through arteries as the vessels are less able to dampen down the kinetic energy from the pressure wave. This is measured using the pulse-wave velocity (PWV), a non-invasive test.[64] It was suggested that measuring vascular health of the aorta and larger vessels could function as a surrogate for early detection of coronary artery dysfunction.[65] PWV is associated with CV mortality, and predicts future development of CVD, in both patients at risk for CVD and the general population.[66-71] Unfortunately, the pulse wave velocity is relatively often studied in the aorta, despite the importance of measuring PWV along the entire arterial tree.[72] Additionally, several risk factors, such as age and an increased heart rate,[72, 73] can influence PWV measurements. There is still uncertainty regarding the reliability of PWV assessment in treatment evaluation and risk prediction,[72, 74] but PWV may indeed add predictive capacity.[66] The role of PWV remains to be further elucidated.

Peripheral function (flow-mediated dilation). Functional alterations in the vascular endothelium precede structural changes, which denotes the protective role of a functionally intact endothelium.[6] Several studies have elucidated the importance of functionally intact endothelium in vasomotor responses to increases in flow and shear.[25, 26] Functional intact endothelium results in vasodilatory responses of coronary arteries following an increase

in blood flow, whilst the presence of atherosclerosis or risk factors impair flow-mediated responses in the coronary circulation.[31, 75, 76] In 1992, Celermajer *et al*. introduced the flow-mediated dilation (FMD), which encompasses non-invasive ultrasound imaging of the brachial artery in response to hyperaemic flow following a brief period of limb occlusion.[7] This reactive hyperaemic flow triggers endothelial cells to produce NO, which in turn results in relaxation of the smooth muscle cells in the vessel wall, causing dilation.[77] In case of endothelial dysfunction, NO production and/or bioavailability becomes impaired, resulting in an impaired dilation in those at risk for cardiovascular disease.[7] Based on these early observations of the presence of vasodilation following elevation in endothelium-dependent flow in both coronary and the brachial artery,[25, 44-46] studies have explored whether peripheral artery responses can be used as a surrogate for coronary vessels. Takase *et al.* examined the possible surrogacy of the brachial artery, by directly evaluating coronary (following coronary angiography) and brachial artery responses to a similar stimulus (i.e. increase in blood flow and shear stress), demonstrating a strong relation between both arteries,[46] similar to others.[44] The past 25 years, the FMD has become a popular technique to examine vascular function in clinical studies. Numerous studies indicate that FMD provides independent prognostic information, exceeding the predictive value of traditional risk factors,[78-84] whilst a more modest association is found in asymptomatic subjects.[83, 85] Nevertheless, a recent comparison of different tests for vascular health in a large cohort of patients at intermediated risk, showed that FMD failed to improve discrimination and risk classification, while FMD reproducibility was quite low in this specific study.[86] Minor adjustments in methodology can critically impact the variation and reproducibility, and whilst clear, stringent methodology and guidelines for valid assessment are available,[19, 84, 87] variation in FMD remains. Therefore, a final judgement on the potential prognostic value of the FMD remains unclear.

Peripheral function (carotid artery reactivity). Whilst the FMD is strongly linked to blood flow (e.g. shear stress) mediated changes in artery diameter, several other stimuli are known to impact the vasculature. One example is activation of the sympathetic nervous system (SNS).[41, 88] The coronary circulation is highly responsive to sympathetic activation (e.g. following a cold pressor test, the immersion of a limb in an ice-bath, CPT), leading to either vasodilation or vasoconstriction, presumably dependent on the integrity of the endothelium.[28, 31] More specifically, vasodilation is typically observed in healthy arteries, whilst paradoxical vasoconstriction is found in irregular arteries with clear presence of atherosclerosis.[28, 89-92] Interestingly, following the cold-pressor test, the coronary arteries behave similarly to the intra-coronary infusion of acetylcholine, the gold standard for coronary artery function.[28, 31] Schächinger *et al.* demonstrated in 147 patients undergoing routine diagnostic following chest pains, that in accordance with the acetylcholine induced coronary responsiveness, the CPT-induced response of the coronary arteries holds strong independent predictive value for future cardiovascular disease.[40, 87]

Several studies have explored the response of the peripheral vasculature to SNS activation, resulting in conflicting observations without clear evidence that the peripheral arteries are highly responsive to SNS stimulation.[93-98] This suggests poor agreement between peripheral and coronary arteries in response to the CPT. In 2000, Rubenfire *et al*. studied patients at risk for CAD and patients with established CAD, and the vasomotor responses following CPT, whilst measuring the carotid artery. Interestingly, the carotid artery, a easily accessible artery, demonstrated marked vasomotor responses to SNS activation.[99] More specifically, it was demonstrated that the carotid artery, similar to the coronary arteries, dilates in healthy volunteers, whilst this dilation was absent or even reversed into constriction in the presence of coronary artery risk factors and/or disease. To date, no previous study explored the possible agreement of coronary and carotid artery responses to the CPT, nor was the potential clinical value of the carotid artery reactivity to assess coronary risk examined.

Outline of this thesis

Aim of the present studies

Measurement of the endothelial function of coronary arteries is a strong predictor for future cardiovascular events. Several studies have explored strategies to measure (peripheral) vascular health to understand the process of development of CVD, but also for early detection of endothelial dysfunction and identification of increased risk for future CVD. The overall aim of my thesis is to explore and improve measures of vascular endothelial function. First, I explored sources of variation in the FMD test. This work was performed to improve current guidelines. Secondly, I focussed on developing an alternative method of measuring endothelial function, the carotid artery reactivity (CAR). This work includes exploring the agreement of the CAR with coronary artery function, the underlying mechanisms and potential prognostic value.

In **part one** of this thesis, I specifically focus on measuring the endothelial function with brachial artery flow-mediated dilation. Although the FMD has been used frequently, the measurement is susceptible to a variety of subject-related and methodological factors influencing its variability. While previous studies have set a specific set of guidelines for the measurement,[84, 87] minor changes in methodological approach may critically impact the variability and reproducibility of the measurement.

Chapter 2 describes the identification of subject- and methodology related factors that contribute to the variation of the brachial artery FMD in a large group of individuals. Accounting for these factors improves reproducibility of the FMD, providing a more accurate measurement of vascular function, reducing measurement error and increasing the power of studies that adopt the FMD. To further explore the importance of adherence to expert guidelines to assess the FMD, **Chapter 3A** depicts the relation between adherence to the current guidelines and reproducibility of the FMD. Furthermore, we examined which subject- and/or methodology-related factors were associated with the reproducibility of the flow-mediated dilation measurement. **Chapter 3B** demonstrates the methods of data acquisition for this study, while in **Chapter 3C** we provide additional support for our statistical analyses.

As impairment of coronary artery blood flow and endothelial function characterizes the development of cardiovascular disease, early detection of endothelial dysfunction remains of utmost importance. A strong prognostic stimulus in assessing coronary function is activation of the sympathetic nervous system. Sympathetic activation can result in either a vasoconstrictive or vasodilatory response of coronary arteries, presumably dependent on the integrity of the endothelium. **Part two** of this thesis focusses on developing and validating a new test, which serves as a surrogate for coronary artery responses to sympathetic stimulation. I explore the potential value of the carotid artery reactivity (CAR) test. The carotid artery demonstrates

similarity with coronary artery responses to a sympathetic stimulus (i.e. a cold pressor test), with a clear vasodilation in both arteries in healthy subjects, whilst this reverses to constriction in those with CV risk factors and/or disease. This similarity in response to the cold pressor test between coronary and carotid arteries is further examined in **Chapter 4**. Furthermore, in this chapter we also assess the relation between the CAR and cardiovascular risk factors. In **Chapter 5** we continue to explore the CAR and we specifically focus on the potential underlying mechanisms mediating the CAR test. First, we explore the responses of the carotid artery to different types of sympathetic stimuli (i.e. the cold pressor test and the lower body negative pressure test). We also examine the underlying mechanisms mediating the vasomotor response by pharmaceutically blocking the α1 receptors, which are believed to be responsible for vasoconstriction during sympathetic activation. In addition, potential similarity between the carotid and coronary artery responses to both stimuli, are examined. Finally, in **Chapter 6** we investigate the potential clinical value by examining prognostic value of the CAR in patients with peripheral arterial disease for the 1-year development of CV events and progression of disease.

Methods applied in this thesis

To examine endothelial function of both the peripheral and central arteries (the brachial, and the carotid and coronary arteries, respectively), vascular structure and different sympathetic stimulation tests, we used different techniques. These different methods will be explained in this section. In accordance with previous set guidelines for vascular ultrasound assessment, all participants attended our measurements in fasted (>6 hours) state, and were instructed to abstain from products high on vitamin C, caffeine, and alcohol (>18 hours), and to not perform strenuous exercise for at least 24 hours.

Brachial artery flow-mediated dilation (FMD). Participants were asked to extend the scanned arm at an angle of ~80 degrees from the torso, following a short (10-15 minutes) resting period in supine position. A rapid inflation and deflation pneumatic cuff (Hokanson, Bellevue (Washington), USA) was positioned on the forearm of the imaged arm distal to the olecranon process to provide a stimulus of forearm ischemia. With an ultrasound system, B-mode images of the brachial artery in the distal third of the upper arm (above the antecubital fossa in the longitudinal plane) were made. When an optimal image was obtained, the ultrasound probe was held stable (manually or by using a clamp) and ultrasound parameters were set to optimise the longitudinal B-mode image. At least one minute of baseline diameter was recorded, after which the pneumatic cuff was inflated to at least 50 mmHg above systolic pressure to occlude arterial inflow for 5 minutes of occlusion. Subsequent cuff deflation induced a brief high-flow (hyperaemic) state that increased wall-shear stress at the brachial artery, causing it to dilate. To assess flow velocity, a mid-artery pulsed Doppler signal was obtained during the protocol.

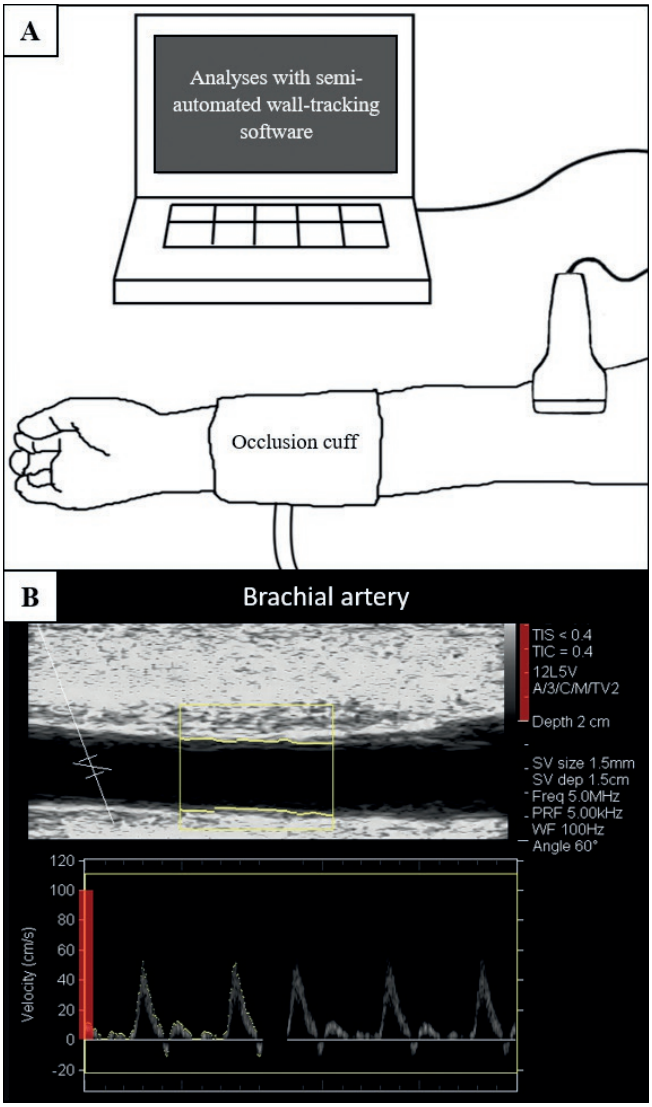


Figure 1. FMD set-up

Panel A shows FMD set-up, with occlusion cuff on the lower arm, and continuous assessment of brachial artery diameter and velocity. Panel B demonstrates the wall-tracking software. The red bars represent the calibration bars. The yellow boxes indicate the region of interest, and the edge-detection feedback.

Carotid artery intima-media thickness (cIMT). Carotid artery intima-media thickness (cIMT) is assessed after a 15 minute rest in supine position. We assessed the baseline resting diameter and wall thickness of the left common carotid artery, approximately 2 cm proximal to the bulbus. The images were optimized by using contrast controls on an ultrasound machine (T3000, Terason, Burlington (Massachusetts), USA), and parameters were set to optimize the B-mode images of the lumen-arterial wall interface. We recorded the IMT continuously for 10 seconds, in 2 different perpendicular planes (differing 90°). From the 2 measurements wall thickness was calculated.

Carotid artery reactivity (CAR). Participants were positioned with the neck extended to allow for assessment of the left common carotid artery. Diameter and red blood cell velocity were recorded continuously during baseline and the sympathetic test with a 10-MHz linear array handheld probe attached to a high resolution ultrasound machine (T3000, Terason, Burlington (Massachusetts), USA). When an optimal image was found, the probe was held stable and the ultrasound parameters were set to optimise the longitudinal, B-mode image of the lumen-arterial wall interface. Continuous pulsed wave Doppler velocity assessments were also obtained and were collected at the lowest possible insonation angle (always <60º).

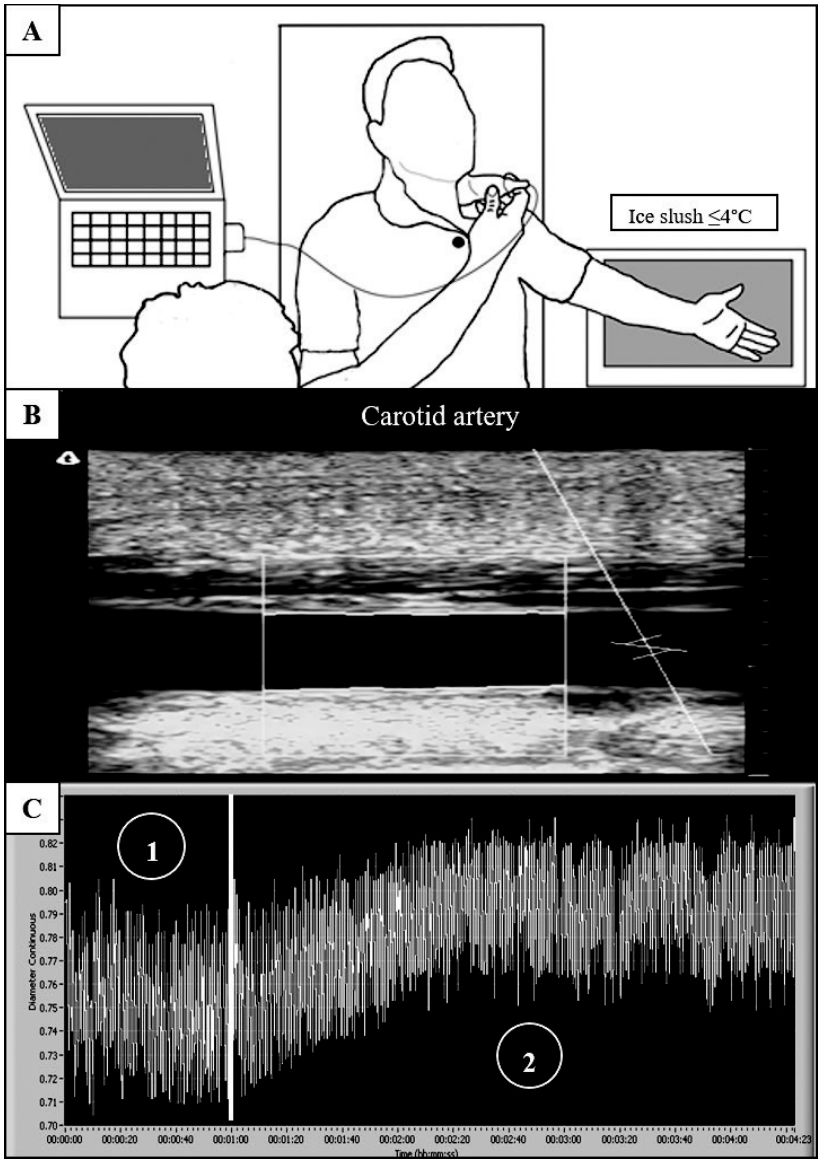


Figure 2. CAR set-up

Panel A demonstrates the CAR set-up, with concomitant echo-Doppler assessment of the carotid artery and a left hand cold pressor test (ice-slush ≤4ºC). Panel B shows the continuous assessment of the carotid artery, using wall-tracking and edge-detection software. Panel C shows a dilatory response during 1; the baseline assessment, and 2; 3-mintues of CPT test.

Coronary artery responses. Before and during sympathetic stimulation, the left anterior descending (LAD, cm) coronary artery velocity was examined using transthoracic ultrasound. This assessment was performed simultaneously with the assessment of the common carotid artery diameter and velocity responses. All echocardiographic measurements were collected by trained sonographers on a commercially available ultrasound system using a transducer (Chapter 4; Vivid Q, GE Medical, Horten, Norway, with a 4 MHz phased array and Chapter 5; Vivid E9; GE, Fairfield, CT, with a broadband M5S 5 MHz or a 3V 3D-array transducer). Participants assumed a left lateral position to allow for data collection. The LAD was imaged using a modified parasternal short axis view from the fourth or fifth left intercostal space, and was assessed using pulsed-wave Doppler. The transducer was positioned such that a 2- to 3-mm segment of the LAD was imaged along the long axis, taking care to align the pulse-wave cursor with the length of the vessel. With a sample volume (2.0 mm) positioned over the colour Doppler signal in the LAD, measurements of the LAD velocity were collected during the sympathetic tests.

Sympathetic stimulation - cold pressor test. The cold pressor test (CPT) consisted of a 3-minute immersion of the left hand in a bucket of ice slush (~4.0 ºC). The participant was positioned supine on a comfortable bed, facilitating arm movement of the left hand into the bucket of ice slush without significant movement of the neck to enable assessment of the carotid artery. After a 1-minute baseline period, the participant immersed the hand up to the wrist in the ice slush for 3 minutes. The participant was instructed not to speak and breathe normally (to prevent hyperventilation) when the hand was submerged into the ice slush.

Sympathetic stimulation - lower body negative pressure test. The participant was positioned in the supine position on a tilt bed, and strapped into a custom-made airtight, lower-body suction chamber at the level of the iliac crest.[100] The lower body negative pressure (LBNP) chamber was then moved from supine position into a left lateral position (~25-30°) to ensure concurrent adequate coronary imaging. The LBNP test consisted of a 5-minute baseline, followed by progressive 2-minute stages, using increments of -10 mmHg, to -80 mmHg or until pre-syncope. LBNP was terminated when a) pre-syncope, defined by a sustained drop in systolic blood pressure <80 mmHg for more than 10 seconds,[101] occurred, or b) on participants request due to the onset of subjective symptoms (e.g. feelings of dizziness, nausea, faintness).

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Let’s get down to business

Part 1

Flow-mediated dilation

Chapter 2

Impact of subject- and methodology-related factors on the reproducibility of brachial artery flow-mediated vasodilation: analysis of 672 individual repeated measurements

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Abstract

Objectives. Brachial artery flow-mediated dilation is a popular technique to examine endothelial function in humans. Identifying subject- and methodological factors related to variation in flow-mediated dilation is important to improve measurement accuracy and applicability.

Methods. Subject- and methodology-related parameters were collected in 672 subjects from 8 affiliated centres world-wide who underwent repeated measures of flow-mediated dilation. All centres adopted contemporary expert-consensus guidelines for flow-mediated dilation assessment. After calculating the coefficient of variation (%) of the flow-mediated dilation for each individual, we constructed quartiles (n=168 per quartile). Based on 2 regression models (1.Subject-related factors, 2.Methodology-related factors), statistically significant components of these two models were added to a final regression model (calculated as β-coefficient and R2). This allowed us to identify factors that independently contributed to the variation in flow-mediated dilation%.

Results. Median coefficient of variation was 17.5%, with healthy volunteers demonstrating a coefficient of variation of 9.3%. Regression models revealed age (β=0.248, P<0.001), hypertension (β=0.104, P<0.001), dyslipidaemia (β=0.331,P<0.001), time between measurements (β=0.318, P<0.001), lab experience (β=-0.133, P<0.001) and baseline flow-mediated dilation% (β=0.082, P<0.05) as contributors to the coefficient of variation. After including all significant factors in the final model, we found that time between measurements, hypertension, baseline flow-mediated dilation%, and lab experience with flow-mediated dilation independently predicted brachial artery variability (total R2=0.202).

Conclusions. Whilst flow-mediated dilation% showed good reproducibility, larger variation was observed in conditions with longer time between measurements, hypertension, less experience and lower baseline flow-mediated dilation%. Accounting for these factors may improve flow-mediated dilation% variability.

Introduction

Cardiovascular disease remains the world's leading cause of morbidity and mortality. Previous studies have provided convincing evidence that endothelial dysfunction is an early manifestation of cardiovascular disease,[1, 2] contributing to development and/or acceleration of the atherosclerotic process. Based on the detrimental role of endothelial dysfunction in this common disease process, studies have attempted to develop and validate (non-invasive) methods and biomarkers to assess endothelial function in humans. The conceptual idea is that identification of endothelial dysfunction, in symptomatic as well as asymptomatic subjects, is related to increased risk for future development of cardiovascular events.[3, 4]

A frequently-used, non-invasive technique to examine endothelial function in humans *in vivo* is flow-mediated dilation (FMD).[5] This measurement adopts high resolution ultrasonography to measure the conduit artery diameter dilatation in response to marked elevation in blood flow (and therefore shear stress) after a 5-minute period of distal limb ischemia.[6] Studies have provided evidence that the FMD-response is endothelium-dependent[7] and largely mediated by nitric oxide,[8] an important and potent vasodilator and anti-atherogenic molecule. The measurement of endothelial function using FMD has become popular in clinically-orientated studies, likely because of its non-invasive nature, ability to predict cardiovascular events[4, 9-11] and correlation to coronary artery endothelial function.[2, 12]

Despite its valid conceptual basis, a number of factors influence the variability of FMD.[13, 14] Previous studies found that FMD is influenced by lifestyle factors (e.g. smoking, physical activity), methodology (e.g. cuff placement, duration of ischemia), intake of food and beverages, hormonal changes, and method of analysis.[8, 11] Although many of these factors are currently being controlled for through adopting expert-consensus guidelines,[11, 15] variation in FMD remains. These sources of variation may be subject-and/or methodology-dependent, but this has not yet been systematically studied. Identification of such factors will contribute to the control of measurement error, which will help to appropriately power studies and aid in the construction of rigorous and standardized guidelines.[11, 16]

The purpose of this study was to identify subject- and methodology-related factors that contribute to FMD variation in humans. To this end, we combined data from previous studies (from 8 research centres) that performed repeated measurements within-subjects of brachial artery FMD in a total of 672 individuals. All included studies were performed according to expert-consensus guidelines.[11] Subsequently, we assessed subject- and methodology-related factors that contributed to brachial artery FMD variability.

Methods

Study population

The International Working Group on Flow-Mediated Dilation (IWG-FMD) originates from eight different research groups in four different countries. All groups provided written consent to contribute their data. We compiled subject-level data from all participating research centres (see supplementary list), including a total of 19 different studies. All affiliated researchers provided details on methodology of included studies in a specifically designed questionnaire. These details were cross-checked with earlier published and/or unpublished data. All centres received an outline of the datasheet, to enhance sufficient and complete data collection. A total of 84 parameters were explored. Data from a total of 672 individuals with measurement of the brachial artery FMD, assessed on at least two separate occasions, obtained by B-mode ultrasound systems were available for data analyses. When studies included more than one repeated measurement, only the first and second measurement were included prior to statistical analyses. All subsequent repeated measurements were rejected, to prevent distortion included parameters.

Brachial artery flow mediated dilation measurements: methodological considerations

We included data from participants whose FMD data were collected on 2 separate occasions without an intervention between both measurements. These measurements were limited to the brachial artery (measurements of e.g. the radial-, femoral or popliteal arteries were excluded), in either the right or left arm (consistent for both measurements). To examine brachial artery FMD, participants extend the scanned arm following a short (10-15 minutes) resting period in the supine position. A rapid inflation and deflation pneumatic cuff was positioned on the forearm of the imaged arm distal to the olecranon process to provide a stimulus of forearm ischemia.[11, 15] With an ultrasound system, B-mode images of the brachial artery in the distal third of the upper arm (above the antecubital fossa in the longitudinal plane) were made. When an optimal image was obtained, the ultrasound probe was held stable (manually or by using a clamp) and ultrasound parameters were set to optimise the longitudinal B-mode image. At least one minute of baseline diameter was recorded, after which the pneumatic cuff was inflated to at least 50 mmHg above systolic pressure to occlude arterial inflow for a standardised length of time (i.e. standardised time of 5 minutes of occlusion). Subsequent cuff deflation induced a brief high-flow (hyperaemic) state that increased wall-shear stress at the brachial artery, causing it to dilate. To assess flow velocity, a mid-artery pulsed Doppler signal was obtained during the protocol.[11, 15] Whilst all study centres used slightly different protocols to collect the repeated FMD measurements, all followed the above described expert-consensus guidelines.

Different types of ultrasound systems were used across the different centres, including; TerasonT3000 (Terason, Aloka, United Kingdom; 10-MHz multifrequency linear array transducer, n=136), Sonos 5500 (Hewlett-Packard, 7.5-MHz linear array transducer, n=20), ESAOTEMyLab25 (ESAOTE, Florence, Italy; 10-MHz linear array transducer, n=54), ESAOTE Picus Just 4D (ESAOTE, Maastricht, the Netherlands, 7.5-MHz linear array transducer, n=60), ESAOTE MyLab^{TM}70 (ESAOTE, Maastricht, the Netherlands; 7.5-MHz linear array transducer, n=51), VIVID E9 (VIVID E9, General Electric, Waukesha, WI, USA, 15-MHz linear array transducer, n=109), AU5 Armonic system (ESAOTE, Florence, Italy; 7.0-MHz linear array transducer, n=136). One included study is a multi-centre study consisting of 7 sub-studies, which used a range of devices (ESAOTE, Philips, Siemens and General Electric, 7.5-10 MHz linear array transducer, n=110).

All studies used (semi)automatic analysis software. However, different software was used across the centres: (1) Custom made MyFMD software, V2012.2, Prof. A.P.G. Hoeks, Department of Biomedical Engineering, Maastricht University, Maastricht, the Netherlands (n=130); (2) Custom made software,[17] Pisa, Italy (n=135); (3) Custom made DICOM software for edge-detection (n=135)[18, 19]; and (4) FMD Studio, Cardiovascular Suite, ClinicalPhysiology, National ResearchCouncil, Pisa, Italy (n=272).[20, 21] All centres collected continuous measurements of the diameter and recorded these (on either VCR or digitally) for post-study analyses. No study used fixed time points for diameter estimation.

Sources of variation

Our primary outcome parameter was the variation between both FMD measurements, for which we calculated the coefficient of variation (CV) for each individual's repeated measurements, calculated as [(sdFMD/meanFMD)*100]. Furthermore, we recorded FMD (%), baseline diameter (cm), maximal diameter (cm), and time between measurements (categorized in <24h, 1-7 days, 8-14 days, 2-4 weeks, or >4 weeks).

Measurement of subject-related factors. We included the following subject-related factors, that were all presented using a continuous scale; age (inclusion ≥18 years, range 18-82 years); weight (range 45-171 kg); height (range 1.55-1.94 m); body mass index (calculated as weight (kg)/ height^2(m), range 17.6-55.8kg/m^2); systolic- and diastolic blood pressure (in mmHg) and calculated mean arterial pressure [MAP, calculated as (2*diastolic pressure + systolic pressure) / 3, range 64-139 mmHg]; and blood-specific parameters (i.e. total cholesterol; high density lipoprotein, HDL; low density lipoprotein, LDL; triglycerides; glucose; all in mmol/L). All original parameters were rescaled to the same metric or most frequently used units (i.e. cholesterol and glucose values converted from mg/dL to mmol/L).[22]

We also presented subject-related factors using categorical scales: sex (male/female); presence of hypertension (conform current guidelines defined as: systolic pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg, or using blood pressure-lowering drugs, yes/no); the presence of diabetes (type 1 or type 2); smoking status (yes/no/history of smoking); presence of dyslipidaemia (yes/no, as specified by each contributing centre), and history and/or presence of cardiovascular disease (CVD).

Measurement of methodology-related parameters. All assessments followed the expert-consensus FMD guidelines, ensuring that the protocol involved cuff placement around the forearm, occlusion for 5-minutes and cuff inflation ≥50 mmHg above systolic pressure. Furthermore, we assessed the following factors; use of a probe holder (yes/no); lab experience (total number of peer-reviewed publications that included measurement of FMD from contributing principal investigator through a Pubmed-based search using the search term "[author] AND flow mediated dilation"); mention of the laboratory's own reported coefficient of variation (mentioned as CV% reported); use of continuous and/or ECG-gated diameter recording; measurement of artery diameter across the cardiac cycle; and the time between measurements (<24h, 1-7 days, 8-14 days, 2-4 weeks, and >4 weeks). The Supplementary material provides details of the questionnaire used to assess these factors.

Missing values

Since missing data were present for all of the 82 individual parameters, we used multiple imputation chained equations to impute parameters. We performed this procedure with a maximum up to 30%, as previously described.[23, 24] Parameters for which 31% or more was data were missing, were excluded from analyses and are not further mentioned. A more detailed outline of the imputation model can be found in the Supplementary material.

Statistical analysis

All data are presented as N(%) or mean ± standard deviation unless stated otherwise. The main outcome measure for the reproducibility of the FMD is the coefficient of variation (CV) calculated for the mean difference between both FMD measurements. All descriptive data were examined in the pooled dataset and in quartiles of variation in FMD (i.e. CV). Based on the CV, we qualified the reproducibility as excellent (0-10%), good (10-20%), moderate (20-30%) or poor (>30%).[25] In multiple linear regression analyses we used the (log transformed) FMD CV as the dependent variable to identify factors that independently contributed to the variability of the FMD measurement, using backward regression analysis. A total of 4 models were constructed; Model 1a - Subject-related factors (continuous scale), Model 1b–Subject-related factors (categorical scale, i.e. presence of hypertension), Model 2–Methodology-related factors, and Model 3–Significant factors from previous models 1a-1b-2. Details of all regression models are given in the Supplemental information. All statistical analyses were

performed using the Statistical Package for Social Sciences, version 20.0 (SPSS, INC. Chicago, IL, USA).

Results

A median CV of 17.5% was observed for the entire population of 672 subjects, whilst a median CV of 9.3% was observed for volunteers without CV risk factors (n=109). We observed substantial variation between subjects regarding the individual CV for the FMD% (Figure 1). When dividing subjects into 4 quartiles, we calculated the CV for each quartile (Mean CV 29.9±46.5, range 0.14-745.33; Median CV Quartile-1 3.25%; Quartile-2 11.74%; Quartile-3 24.76%; Quartile-4 61.03%). We found an excellent, good or moderate CV in 33% (n=221), 22% (n=147), and 14% (n=94) of the sample, respectively. A poor CV was observed in 31% of the cases (n=210).

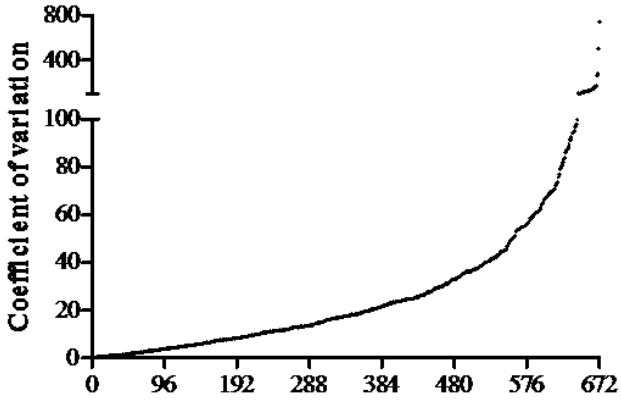

Figure 1. Individual reproducibility in Brachial artery FMD
Data of all subjects (n=672) relating to the individual reproducibility of the brachial artery FMD across 2 repeated measurements.

Subject-related factors

Age, BMI, total cholesterol, and glucose levels showed a gradual increase across quartiles, with Q3 and Q4 (i.e. large variation in FMD) showing significantly higher values than Q1 (Table 1). Systolic, diastolic and mean blood pressure were highest in Q2-3, whilst this difference was lost in Q4 (Table 1). When subject-related factors were presented using a categorical scale, hypertension and dyslipidaemia had significant impact on the reproducibility of the FMD (presence of hypertension Q1 15.5%, Q2 30.4%, Q3 32.1% and Q4 21.4%, diabetes

Q1 0%, Q2 0%, Q3 1.2% and Q4 0.6%, both P<0.001), but not sex, smoking status, diabetes mellitus and CVD.

Methodology-related factors

FMD% and baseline diameter were significantly different across quartiles of the CV (Table 2). Subject in Q4 had a lower FMD and a larger baseline diameter (Table 2). We found that all factors related to the practical performance of the FMD, except the use of a probe holder, were significantly different between quartiles (Table 2). Larger variation in CV FMD% (i.e. Q3-4) was associated with absence of ECG-gated recording, no measurement of the diameter across the cardiac cycle, longer time between tests, less experience of the research centre in FMD measurements, and absence of reporting the CV of the laboratory in manuscripts (Table 2).

Table 2. Subject-related factors.

Continuous scale	Pooled {29.9±46.5}	Quartile 1 {3.25%}	Quartile 2 {11.74%}	Quartile 3 {24.76%}	Quartile 4 {61.03%}	P-value
Age (years)	46±17 (*655*)	40±16 *163*	42±15 *164*	46±16* *164*	54±16* *164*	<0.001
Sex (% male)	66 *671*	64 *168*	67 *168*	68 *167*	67 *168*	0.895
Weight (kg)	77.4±13.1 *636*	75.9±12.1 *163*	76.7±11.8 *161*	78.6±14.4 *160*	78.3±14.1 *152*	0.210
Height (cm)	1.75±0.1 *637*	1.76±0.1 *163*	1.76±0.1 *161*	1.75±0.1 *160*	1.75±0.1 *152*	0.657
BMI (kg/m)	25.3±3.7 *657*	24.6±3.4 *164*	24.9±3.3 *165*	25.8±4.2* *164*	25.9±3.5* *164*	0.003
Systolic BP (mmHg)	129±15 *645*	127±13 *161*	131±14* *163*	130±16* *159*	128±15 *162*	0.023
Diastolic BP (mmHg)	79±11 *645*	78±11 *161*	81±12* *163*	79±12 *159*	76±11 *162*	<0.001
Mean BP (mmHg)	96±12 *655*	94±11 *135*	98±12* *165*	96±13 *163*	94±11 *164*	0.002
Cholesterol (mmol/L)	5.3±1.0 *544*	5.1±1.0 *135*	5.2±1.0 *134*	5.4±1.0* *134*	5.6±0.9* *141*	<0.001
HDL (mmol/L)	1.4±0.4 *508*	1.4±0.3 *127*	1.4 ±0.3 *126*	1.4±0.3 *124*	1.4±0.4 *131*	0.414
LDL (mmol/L)	3.5±0.8 *466*	3.3±0.8 *115*	3.3±0.8 *109*	3.5±0.9* *112*	3.7±0.8* *130*	<0.001
Triglycerides (mmol/L)	1.4±1 *529*	1.3±0.8 *129*	1.4±1.3 *130*	1.4±0.9 *130*	1.3±0.8 *140*	0.924
Glucose (mmol/L)	5.1±0.7 *466*	5.0±0.7 *132*	5.0±0.9 *132*	5.0±0.7 *114*	5.4±0.7* *88*	<0.001

Subject-related factors for whole group (n=672) and quartiles (of n=168 each) with median CV reported per quartile. Data are reported as mean ± SD with total number of subjects available for analysis presented below in italic. P-value refers to an ANOVA. *Post-hoc significantly different from Quartile 1 at P<0.05.

Table 2. Methodological-related factors

Continuous scale	**Pooled {29.9±46.5}**	**Quartile 1 {3.25%}**	**Quartile 2 {11.74%}**	**Quartile 3 {24.76%}**	**Quartile 4 {61.03%}**	**P-value**
Baseline diameter (mm)	4.3±0.8 *672*	4.1±0.8 *168*	4.3±0.7* *168*	4.4±0.8* *168*	4.4±0.8* *168*	<0.001
Maximal diameter (mm)	4.5±0.8 *672*	4.3±0.8 *168*	4.5±0.7* *168*	4.6±0.9* *168*	4.5±0.8* *168*	<0.001
FMD (%)	5.4±3.0 *672*	6.1±2.8 *168*	5.8±2.4 *168*	5.7±2.8 *168*	4.1±3.6* *168*	<0.001
Laboratory experience (papers per PI)	29.2±24.8 *672*	35.6±21.9 *168*	35.1±22.9 *168*	30.9±25.3* *168*	15.4±23.6* *168*	<0.001
CV reported (%)	16.8±9.5 *612*	14.7±6.9 *155*	14.6±6.7 *160*	16.5±9.5 *158*	22.2±12.4 *139*	<0.001
Categorial scale						
Analysis by laboratory	96 *672*	99 *168*	99 *168*	95* *168*	92* *168*	<0.001
ECG-gated recording	28 *672*	25 *168*	38* *168*	35* *168*	13* *168*	<0.001
Cardiac cycle (%)	84 *672*	87 *168*	88 *168*	87 *168*	73* *168*	<0.001
Probe holder (%)	80 *672*	77 *168*	79 *168*	77 *168*	86 *168*	0.110
Time: <24 hours (%)	53	69	69	52	21	<0.001
1-7 days (%)	6	6	9	6	4	
8-14 days (%)	7	5	5	10	8	
2-4 weeks (%)	9	9	6	8	11	
>4weeks (%)	25	11	11	24	56	
	672	*168*	*168*	*168*	*168*	

Methodological-related factors presented for whole group (n=672) and quartiles (n=168 each) with median CV reported per quartile. Data are reported as mean ± SD with the total number of subjects available for analysis presented below in italic. P-value refers to an ANOVA. *Post-hoc significantly different from Quartile 1 at P<0.05.

Regression analyses

Model 1a – Subject-related factors (continuous). After including all subject-related factors that significantly differed across quartiles, this model showed an R^2=0.087 and adjusted R^2=0.086. We found that only age predicted variation in FMD%CV (β=0.248, ratio of 28.1%, CI[0.020-0.035], *p-value* <0.001).

Model 1b–Subject-related factors (categorical). Including all subject-related factors that differed across quartiles, we found an R^2=0.112 and adjusted R^2=0.108. We identified hypertension (β=0.104, ratio of 11%, CI [0.095-0.533], *p-value* 0.005), dyslipidaemia (β=0.331, ratio of 39.2%, CI [0.813-1.275], *p-value* <0.001) and sex (β=-0.069, ratio of -6.7%, CI[-0.390-0.010], *p-value* 0.063) as significant predictors for the reproducibility of the FMD%.

Model 2–Methodology-related factors. This model showed an R^2=0.198 and adjusted R^2=0.184 when including methodology-related factors that differed across quartiles. The model identified time between measurements (β=0.318, ratio of 37.5%,CI[0.179-0.298], *p-value <0.001*), FMD% at baseline (β=-0.124, ratio of -11.7%, CI [-0.098--0.021], *p-value* 0.002), baseline diameter (β=0.082, ratio of 8.6%, CI[0.007-0.270], *p-value* 0.039) and lab experience (β=-0.133, ratio of -12.4%, CI [-0.011--0.003], *p-value* 0.001) as significant contributors to the variation in FMD% CV.

Model 3 - Overall model Factors identified by models 1a, 1b and 2 were included in the overall model which resulted in an R^2=0.208 and adjusted R^2=0.202. Backward linear regression analysis identified time between measurements (β=0.291, ratio of 33.8%, CI [0.156-0.273], *p-value* <0.001), hypertension (β=0.096, ratio of 10.1%, CI[0.068-0.501], *p-value* 0.010), baseline FMD% (β=-0.142, ratio of -13.3%, CI [-0.105--0.030], *p-value* <0.001) and lab experience (β=-0.131, ratio of -12.3%, CI [-0.012--0.003], *p-value* 0.001) as significant contributors to the variation in FMD% across 2 repeated measurements (Figure 2). Baseline diameter demonstrated a borderline significant association with FMD% reproducibility (β=0.070, ratio of 7.2%, CI [-0.015-0.242], *p-value* 0.084).

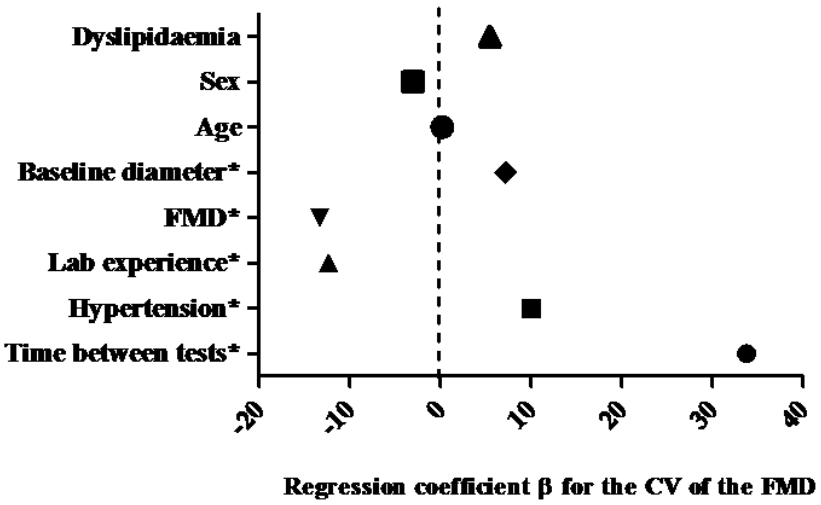

Figure 2. Regression analysis
Plot for regression coefficient β for the coefficient of variation (CV) of the flow mediated dilation (FMD). * implies a statistical significant contribution in final model

Discussion

This study included 672 repeated measurement of the brachial artery FMD, involving data from different research centres and various populations. This allowed us to comprehensively explore factors contributing to the within-subject variability of brachial artery FMD%, when measured according contemporary guidelines.[11] We present the following observations. First, the majority of the measurements showed an excellent-to-good reproducibility. For asymptomatic subjects, the median CV was 9.3%. This demonstrates that FMD is a reproducible tool to assess endothelial function *in vivo*. Secondly, we also found substantial variation between individuals in the CV of FMD%. In particular, the presence of hypertension contributed to a larger variation in FMD%, independent of other factors. Third, we found that a poorer reproducibility of the FMD was associated with the presence of a lower baseline FMD%, a higher baseline brachial artery diameter, a longer time period between repeated measurements, and less experience of the laboratory with the FMD measurement. Taking these factors into consideration for sample size calculations in future studies will help to decrease chances of type II errors.

Subject-related factors

Several previous studies have explored and described reproducibility of brachial artery FMD and presented mixed results, ranging from an excellent to poor reproducibility.[13, 26, 27] The overall median CV% in our analysis of 17.5% in the whole study population, and 9.3% in subjects without CV risk/disease, are in line with findings of most previous studies that reported a good reproducibility.[14, 16, 28-30] An important strength of our analysis is the large number of repeated measurements, which allowed us to identify between-subject and – laboratory related factors contributing to the variation in brachial artery FMD% within an individual. Interestingly, we found that older age, dyslipidaemia and presence of hypertension were related to larger variation in FMD%. This suggests, in agreement with previous work,[28] that reproducibility of the FMD may be lower in populations with clinical symptoms than in healthy, young subjects.

An explanation for the larger variation in clinical populations could be the presence of a lower baseline FMD% that is typically observed in older subjects[31] and in those with hypertension,[32] CVD[33] or dyslipidaemia.[14] Indeed, we found that baseline FMD% is a strong and independent predictor for larger variability. Therefore, baseline FMD% was added to the statistical model to explore its impact on variability in FMD% independent of older age, hypertension and dyslipidaemia. Interestingly, in this model the impact of age and dyslipidaemia disappeared, suggesting that the lower baseline FMD% in older subjects is at least partly responsible for the larger variation with increasing age. In contrast, the impact of hypertension remained significant, indicating that other factors play a role in the larger variation in repeated measurements of brachial artery FMD%. Possibly, this poorer reproducibility may relate to higher stiffness of the vessels in clinical populations, compared to healthy volunteers.[34] Craiem *et al.* also found that subjects with CVD, despite comparable baseline FMD% values, demonstrate a larger coefficient of variation compared to healthy controls.[28]

Methodology-related factors

Identification of methodology-related factors that contribute to the variation in FMD is highly relevant because such factors can potentially be controlled for. Several previous studies have highlighted the importance of methodological factors, which formed the basis for the FMD expert consensus guidelines.[11] The present study identified time between measurements and lab experience as independent determinants of the variation in FMD%, with more time between FMD measurements leading to a higher CV. Most studies that explored FMD reproducibility included fixed time points between measurements, which makes direct comparisons of the duration between testing difficult. Interestingly, Charakida *et al.* explored FMD reproducibility after a few hours, 2 day, 3 months and 9 month.[35] In agreement with our findings, this study also demonstrate a poorer CV with increased time between re-testing. In contrast, Sorensen *et al.* found no difference in reproducibility when FMD was

repeated after 1-2 days, 1-2 weeks or 2-4 months.[27] However, this study did not apply FMD measurements according to current guidelines, which may have affected the results. Whilst longer time between repeated measures may be associated with increased variability due to purely methodological variation, it is also likely that true biological variability is greater under circumstances where the repeated measure is more distant in time.

Laboratories that provided data for this analysis adopted expert consensus guidelines to perform and analyse FMD. This makes it difficult to explore the importance, for reproducibility, of the individual aspects within these guidelines. Nonetheless, our analysis showed that laboratory experience with FMD measurements independently contributes to the variation in FMD measurement. More specifically, the greater the experience of a laboratory with the FMD technique, the smaller the variation between repeated FMD measurements. This somewhat self-evident finding is nonetheless important, as it should guide laboratories who adopt the technique in attaining the level of practice and experience required before robust measures can be assumed. Nonetheless, limited experience of FMD did not completely invalidate assessment: the subgroup of healthy subjects without CV risk/disease that showed a CV of 9.3±19% (n=109) included data from both experienced and less experienced laboratories, demonstrating the feasibility of a low CV in FMD measurements. This is in accordance with previous multi-centre studies.[16] These data demonstrate the importance of adherence to the expert-consensus guidelines in addition to *a priori practice and* experience with the FMD-technique.

Practical relevance. This study demonstrates that, in addition to adopting current guidelines, some factors should be considered that might affect the variation of the FMD. For example, larger FMD reproducibility is observed when the time between measurements increases and/or in the presence of hypertension, and low resting FMD%. These factors should be taken into consideration when performing a sample size calculation and in the design of the study. Furthermore, the data of this study also emphasise that, in addition to fair reproducibility of the FMD in less experienced laboratories, training and gaining more experience is likely to minimise measurement error of the FMD-technique.

Limitations. One limitation of our study is that it was not prospectively designed to address FMD reproducibility. This may have introduced some error, especially relating to controlling physical activity and/or dietary instructions for the time between testing. However, all data was collected as in a 'real world' study rather than being set-up as a reproducibility study. Therefore, our study possesses ecological validity and can be extrapolated to various research settings. Another limitation is that all data in our analysis derive from laboratories adopting current guidelines for FMD measurement. Therefore, we were unable to address the relative importance of individual aspects included in these guidelines. In addition, whilst all centres

indicated they adhered to the expert-consensus guidelines, we have no specific data on the internal control of adherence and/or small variation within these guidelines between centres (e.g. differences in analysis software, ultrasound machines). Such differences may in part contribute to the inherent variability of the FMD.

In conclusion, we have shown in a large dataset of repeated measurements that the majority of FMD measurements show an excellent-to-moderate reproducibility. Despite adopting expert consensus guidelines, several subject and methodology-related factors have independent impact on the variation in FMD% between two measurements. These include the presence of hypertension, a lower resting FMD%, a larger baseline artery diameter, a longer time between subsequent measurements, and less laboratory experience with the measurement. Future studies should take these subject- and methodology-related factors into consideration to improve sample size calculation. Such procedures will importantly decrease variability of the FMD and, consequently, decrease chances for type II errors in studies that rely on FMD as their primary outcome parameter.

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Supplementary data – Study contributions

All included studies involved in the van Mil *et al.* are listed below, grouped per country on alphabetical order, with contributing author(s).

Australia	**University of Western Australia - prof. Daniel Green**
1 study	dr. Ceri Atkinson, dr. Louise Naylor and dr. Howard Carter
Italy	**Università di Pisa - prof. Lorenzo Ghiadoni**
3 studies	dr. Rosa Maria Bruno
The Netherlands	**Maastricht University - prof. Ronald Mensink & dr. Koen Reesink**
1 study	dr. Frank van Bussel and dr. Yvo Kusters (CARIM)
1 study	dr. Peter Joris (NUTRIM)
	Radboudumc Nijmegen - prof. Dick Thijssen
2 studies	dr. Tim Schreuder
1 study	drs. Joost Seeger
1 study	dr. Constatijn Wouters
1 study	prof. Dick Thijssen
	Unilever - dr. Peter Zock
2 studies	dr. Arno Greyling
	Wageningen University - prof. Marianne Gelijnse
1 study	dr. Lieke Gijsbers
1 study	dr. James Dower
The United Kingdom	**Liverpool John Moore's University - prof. Dick Thijssen and prof. Daniel Green**
2 studies	dr. Gurpreet Birk
2 studies	dr. Tom Bailey

Supplementary data - questionnaire

This Supplementary data contains the methodological questionnaire we asked all contributing centres (and authors) to fill in, to acquire the necessary methodological information.

Study name :	
Number of subjects included in the study:	

1. Investigators	
Please provide below the names and e-mail addresses of all investigators responsible for your study who should be mentioned as co-authors in this project	
Name	*E-mail address*

1. Study information
Please give a short description of the study population below (e.g. apparently healthy, specific age range, obese individuals, hypertensive individuals, etc.)
Please list all inclusion and exclusion criteria for the study below
Please list all references to publications containing results of this study below

1. Diameter/Flow Mediated Dilation measurements of the brachial artery	
Please give a short description of the subject preparation prior to the *FMD* measurement protocol below (i.e. resting, refrained from smoking/alcohol/exercise or not, vasoactive medications withheld or not, position of the participant, etc.)	
Please give a short description of the post occlusive reactive hyperemia protocol below (i.e. brachial artery occluded distal or proximal to the ultrasound probe, occlusion cuff pressure, duration of occlusion, etc.)	
Which device was used for obtaining B-mode images of the brachial artery (i.e. name, version, type & frequency of probe, etc.)	

(Semi)Automated analysis using edge detection and wall tracking software? If yes, please provide details of the system used (i.e. name, version, software version, etc.)	Yes: ☐ No: ☐
Continuous measurement of diameter? If yes, what was the size of the time bins (sec)?	Yes: ☐ No: ☐
Diameter measured at fixed time points? If yes, at which time points?	Yes: ☐ No: ☐
Were diameter measurements ECG-gated?	Yes: ☐ No: ☐
Were diameter measurements averaged over the cardiac cycle?	Yes: ☐ No: ☐
Were FMD measurements centrally analyzed?	Yes: ☐ No: ☐
Simultaneous acquisition of pulse-wave Doppler velocity signal for quantification of shear stimulus? If yes, what was the insonation angle and how was the shear stimulus defined?	Yes: ☐ No: ☐
Was a stereotactic probe-holder used	Yes: ☐ No: ☐
What was the time duration between repeated measurements?	
What was the CV of the lab for %FMD at the time of the study?	
What was the level of the sonographer's experience at the time of the study? (i.e. number of years/ number of measurements conducted)	
Was endothelium-independent dilation measured? If yes, please provide details of the protocol used (i.e. dose of GTN, duration of measurement, etc.)	Yes: ☐ No: ☐

Supplementary data – imputation model

This Supplementary data provides a more detailed description of the imputation model used to impute missing values. Parameters for which 31% or more was data were missing, were excluded from analyses and are not further mentioned. This model was developed in collaboration with dr. Lian van Engelen, who had previous experience with related data assessments. All analyses were done in SPSS 20.0, as described earlier.

MULTIPLE IMPUTATION weight height BMI SBP DBP MAP t_chol ldl_chol hdl_chol diabetes glucose lntrig
lnage lnFMD_CV hypertension_control mean_FMD stdev_FMD time dyslipidaemia smoking_1 smoking_2
smoking_3 smoking
/IMPUTE METHOD=AUTO NIMPUTATIONS=10 MAXPCTMISSING=NONE MAXCASEDRAWS=50 MAXPARAMDRAWS=2
/CONSTRAINTS weight(MIN=40.0 MAX=175.0)
/CONSTRAINTS height(MIN=1.45 MAX=2.1)
/CONSTRAINTS BMI(MIN=15.0 MAX=57.0)
/CONSTRAINTS SBP(MIN=80.0 MAX=200.0)
/CONSTRAINTS DBP(MIN=40.0 MAX=120.0)
/CONSTRAINTS MAP(MIN=60.0 MAX=145.0)
/CONSTRAINTS t_chol(MIN=0 MAX=12)
/CONSTRAINTS ldl_chol(MIN=0.0 MAX=10.0)
/CONSTRAINTS hdl_chol(MIN=0.0 MAX=5.0)
/CONSTRAINTS glucose(MIN=0.0 MAX=12.0)
/CONSTRAINTS lntrig(MIN=-2.0 MAX=3.0)
/CONSTRAINTS lnage(ROLE=IND)
/CONSTRAINTS lnFMD_CV(ROLE=IND)
/CONSTRAINTS hypertension_control(ROLE=IND)
/CONSTRAINTS mean_FMD(ROLE=IND)
/CONSTRAINTS stdev_FMD(ROLE=IND)
/CONSTRAINTS time(ROLE=IND)
/CONSTRAINTS smoking_1(ROLE=IND)
/CONSTRAINTS smoking_2(ROLE=IND)
/CONSTRAINTS smoking_3(ROLE=IND)
/MISSINGSUMMARIES NONE
/IMPUTATIONSUMMARIES MODELS DESCRIPTIVES
/OUTFILE IMPUTATIONS=IWG_imputation_total .

To briefly elaborate on the model above, we imputed missing data for; weight, height, BMI, SBP, DBP, MAP, t_chol, ldl_chol, hdl_chol, diabetes, glucose, and lntrig. To provide for enough possible data points, we used; lnage, lnFMD_CV, hypertension_control, mean_FMD, stdev_FMD, time, dyslipidaemia, smoking_1, smoking_2, and smoking_3 as independent predictors (ROLE=IND).

For all imputed data we set constraints on reasonable values, to assure that the imputations would be possible within the normal distributional range.

Chapter 3a

Adherence to guidelines strongly improves reproducibility of brachial artery flow-mediated dilation

Arno Greyling
Anke C.C.M. van Mil
Peter L. Zock
Daniel J. Green
Lorenzo Ghiadoni
Dick H. Thijssen

Atherosclerosis. 2016

Abstract

Background. Brachial artery FMD is widely used as a non-invasive measure of endothelial function. Adherence to expert guidelines is believed to be of vital importance to obtain reproducible measurements. We conducted a systematic review of studies reporting on the reproducibility of the FMD in order to determine the relation between adherence to current expert guidelines for FMD measurement and its reproducibility.

Methods. Medline-database was searched through July 2015 and 458 records were screened for FMD reproducibility studies reporting the mean difference and variance of repeated FMD measurements. An adherence score was assigned to each of the included studies based on reported adherence to published guidelines on the assessment of brachial artery FMD. A Typical Error Estimate (TEE) of the FMD was calculated for each included study. The relation between the FMD TEE and the adherence score was investigated by means of Pearson correlation coefficients and multiple linear regression analysis.

Results. Twenty-seven studies involving 48 study groups and 1,537 subjects were included in the analyses. The adherence score ranged from 2.4 to 9.2 (out of a maximum of 10) and was strongly and inversely correlated with FMD TEE (adjusted $R2=0.36$, $P<0.01$). Use of automated edge-detection software, continuous diameter measurement, true peak diameter for %FMD calculation, a stereotactic probe holder, and higher age emerged as factors associated with a lower FMD TEE.

Conclusions. These data demonstrate that adherence to current expert consensus guidelines and applying contemporary techniques for measuring brachial artery FMD decreases its measurement error.

Introduction

The endothelium is a key regulator of vascular homeostasis and endothelial dysfunction is an early manifestation of atherosclerosis.[1] Currently, the most widely used technique to study endothelial function *in vivo* is the flow-mediated dilation (FMD) of the brachial artery. This is a non-invasive, ultrasound-based method which correlates with endothelial function of the coronary arteries[2, 3] and independently predicts cardiovascular disease (CVD).[4, 5] The technique is attractive as a surrogate end-point, especially since changes in FMD can be detected across a relatively short timeframe.[6] Despite its popularity, minor changes in the methodological approach may critically impact variability and decrease reproducibility of the FMD response.[7-9]

Previous expert consensus guidelines have made important contributions to standardize the technical approach and to set minimum standard requirements for FMD measurements.[10, 11] However, not all studies on FMD apply these recommendations, or only in part. The impact of adherence to these guidelines on the reproducibility of FMD measurements is currently unclear, but may importantly contribute to the measurement error of the FMD technique Furthermore, little is known about the relative importance of the individual aspects of the expert-consensus guidelines to contribute to the reproducibility of the FMD. Better quantitative data on this matter can help reduce variation within and between studies, which will increase the statistical power of studies on FMD to detect changes and, subsequently, decrease chances for type II errors.

In light of these considerations, we hypothesized that adherence to expert consensus guidelines is related to better reproducibility of FMD measurements.[10, 11] Therefore, we performed a systematic search for published studies that reported data on reproducibility of FMD measurements, and investigated the relation between (full or partial) adherence to current expert consensus guidelines and reproducibility of the FMD. Secondly, we explored which subject- and methodology-related factors were related to FMD reproducibility.

Methods

Search Strategy

The MEDLINE bibliographic database was searched (January 2000 through July 2015) for studies that assessed the reproducibility of the FMD using the following search terms: *"flow mediated dilation", "flow mediated dilatation", "flow mediated vasodilation", "flow mediated vasodilatation", "endothelial function", "endothelial dysfunction", "FMD", "FMV", "brachial artery", "reproducibility", "reliability", "repeatability", "coefficient of variation",*

"CV", and "variance". The search was limited to studies in human adults published in the English language. Additionally, we supplemented the search by hand-searching references of included studies and relevant reviews and meta-analyses on this topic.

Selection of Studies

Included studies were identified by means of a two-step selection process. During the first step, two reviewers (ACCMvM, AG) independently screened titles, abstracts and keywords of publications to identify potentially eligible studies. Studies were included if the mean difference and variance of repeated FMD measurements of the brachial artery were reported. During step 2 of the selection, both reviewers examined the full text of these publications to gauge eligibility based on two additional inclusion criteria: FMD was determined through non-invasive ultrasound imaging, and a reactive hyperaemia protocol (with an ischemia duration of 4 to 5 minutes) was used to elicit the shear stress stimulus required for FMD. Thus, studies that adopted (ischemic) hand-grip exercise, passive movement and/or skin warming protocols to elicit (brachial) artery dilation were not included in our analysis. In cases of discrepancy between the reviewers, eligibility was discussed along with a third reviewer (DHJT) until consensus was reached.

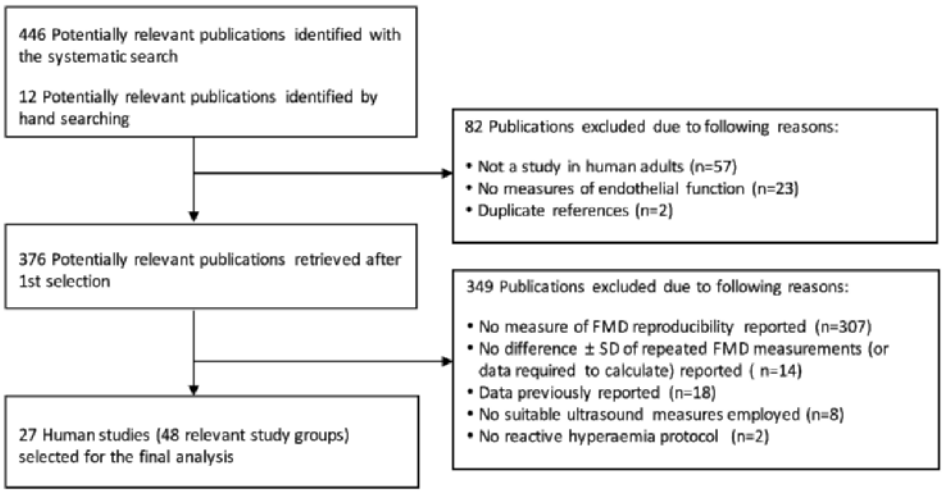

Figure 1. Flow diagram of the study selection procedure.

Data extraction

Study and subject characteristics: A standardized data collection sheet was used to extract general publication details (author, year of publication, country) and specific study- and subject characteristics: number of subjects, mean age (in years); CVD risk status of the study population (defined as presence of diagnosed CVD, hypertension or diabetes); baseline brachial artery diameter (in mm); % brachial artery FMD and its associated variance for each repeated measurement; and the mean absolute difference between repeated FMD measurements and its associated variance.

Adherence to guidelines. We extracted information from the methods sections of the individual papers to assess the adherence to current expert-consensus guidelines. Based on recent guidelines,[11] we scored each individual study on the reporting of 19 different factors which were divided over 4 categories. The categories were related to: 1. Subject preparation (10 items), 2. Image acquisition (4 items), 3. Data analysis (3 items), and 4. Laboratory (2 items). Before performing the systematic literature search, values were assigned to each factor proportional to its perceived importance for valid assessment of the FMD. This was done through expert consensus discussion within the Working Group (AG, LG and DHJT).[12] The *"Adherence Score"* that could be assigned to a study ranged from 0 to 10 points, depending on how many of the 19 different factors that were reported. In addition, we counted the number of previous studies on FMD published by the principal author of each study included in the systematic review. This number served as a measure of the perceived experience in FMD measurements for each centre at the time of publication of the reproducibility data included here.

Table 1. General characteristics of the FMD reproducibility studies included in the systematic review.

Source	*Health status*	*Number of subjects*	*Age (years)*	*Mean baseline FMD (%)*	*Mean baseline diameter (mm)*	*Time between measurements**	*TEE*
Kanahara 2014[14]	Healthy	32	40	7.90	3.83	14	1.28
Charakida 2013[15]	CVD, Diabetes	67	61	4.10	4.55	2	0.94
Charakida 2013		67	61	4.10	4.60	90	1.04
Charakida 2013		67	61	4.10	4.65	270	1.47
Ghiadoni 2012[16]	Healthy	135	32	6.52	3.53	1 hour	0.83
Ghiadoni 2012		135	32	6.52	3.55	30	1.15
Onkelinx 2012[17]	CVD	18	68	6.80	3.92	0.5 hour	0.94
Onkelinx 2012		18	68	7.13	3.91	2	0.88
Lima 2010[18]	Healthy	31	25	13.17	3.57	2	2.91
Thijssen 2009[19]	Healthy	10	24	6.83	4.28	0.5 hour	0.89
Donald 2008[20]	Healthy	32	43	8.10	3.70	6 hours	0.79
Donald 2008[γ]		34	43	7.50	3.70	7	0.79
Donald 2008[γ]		37	43	8.10	3.75	30	0.53
Donald 2008[γ]		35	43	7.80	3.80	90	0.74
Donald 2008[γ]		32	43	7.30	3.70	6 hours	1.08
Donald 2008**		34	43	6.70	3.70	7	0.95
Donald 2008**		37	43	7.50	3.75	30	0.63
Donald 2008**		35	43	7.10	3.80	90	0.87
Simova 2008[21]	CVD, Hypertension	40	62	6.05	3.84	0.25 hour	0.85
Craiem 2007[22]	Healthy	10	32	7.60	3.95	1 hour	0.80
Craiem 2007		10	32	8.10	3.89	7	0.91
Craiem 2007	CVD	26	44	6.98	3.97	1 hour	1.34
Craiem 2007		26	44	5.66	4.15	30	0.96
Harris 2007[23]	Healthy	9	57	7.80	4.11	2	1.32
Meirelles 2007[24]	Healthy	10	33	19.90	3.50	1.5 hours	2.70
Meirelles 2007		13	33	16.50	3.55	3	2.50
Donald 2006[25]	Healthy	16	28	7.30	3.55	1	1.63
Harris 2006[26]	Healthy	16	23	9.88	3.74	2 hours	0.71
Leeson 2006[27]	Healthy	17	32	4.74	4.05	20	1.22
Elsen 2005[28]	Healthy	15	23	4.61	4.04	1	0.63
Sejda 2005[29]	Healthy	18	28	5.95	4.04	7	3.89
Sejda 2005		18	28	4.23	4.15	7	1.63
Stoner 2004[30]	Healthy	9	23	10.20	3.90	2	3.26
West 2004[31]	Diabetes	18	55	5.57	4.01	7	0.81
West 2004		18	55	5.57	4.01	14	1.07

Source	*Health status*	*Number of subjects*	*Age (years)*	*Mean baseline FMD (%)*	*Mean baseline diameter (mm)*	*Time between measurements**	*TEE*
Sidhu 2002[32]	Healthy	12	36	5.38	3.94	20	0.37
Sidhu 2002	CVD	12	62	1.80	4.29	20	0.33
Beux 2001[33]	Healthy	38	44	6.62	4.41	1 hour	1.97
Beux 2001		38	44	4.32	4.41	1 hour	1.22
De Roos 2001[34]	Healthy	34	27	4.13	3.90	25	2.01
Herrington 2001[35]	Healthy	127	79	2.63	4.53	7	0.79
Herrington 2001		30	45	7.87	4.35	7	1.46
Woodman 2001[36]	Healthy	24	55	6.60	4.06	7	0.71
Lind 2000[37]	Healthy	10	22	7.40	3.55	2 hours	2.19
Lind 2000		10	22	7.40	3.55	21	2.82
Preik 2000[38]	Healthy	8	28	10.60	3.62	20	1.06
Liang 1998[39]	Healthy	30	44	10.80	3.84	18	2.01
Hardie 1997[40]	Healthy	19	36	3.00	3.78	90	4.83

*measured in days, unless stated otherwise, ᵞtrue peak diameter; **peak at 60 seconds

Statistical analysis

Reported measures of FMD reproducibility varied between studies. Many studies presented the coefficient of variation (CV) of repeated measurements, although this measure was calculated in a number of different ways, precluding direct comparisons. Measures of reproducibility included the technical error of the measurement (TEM), Pearson- and intraclass correlation coefficients (ICC), and limits of agreement. In order to make valid comparisons between studies, we defined as primary outcome measure the typical error of estimate (TEE) of FMD, which is calculated as standard deviation of the paired differences/√2.[13]

Data are presented as mean ± SD or median (range) as appropriate for continuous variables and as frequencies for categorical variables. FMD TEE data were highly skewed (Shapiro-Wilk test, $P<0.0001$) and were log transformed prior to the analyses. Relations between log-FMD TEE and continuous variables were determined by Pearson correlation coefficients analysis. For categorical variables, the statistical significance of differences in FMD TEE between different levels were assessed by the Mann-Whitney *U* test. Significant correlates were entered in a multivariate linear regression analyses with backward elimination to identify independent predictors of FMD TEE. All analyses were conducted using JMP version 11.0 (SAS Institute Inc., Cary, NC, USA).

Results

Our systematic search identified 446 potentially relevant publications and an additional 12 were obtained through review of references of included studies, relevant reviews and meta-analyses. Twenty-seven studies[14-40] with 48 relevant study groups met our inclusion criteria and were included in our analysis (Figure 1). Characteristics of the included study groups are presented in Table 1. The 48 study groups comprised a total of 1,537 subjects (mean sample size 32; range, 8-135) with a mean age of 41.5 years (range, 22-79 years). Eleven study groups included subjects with increased CVD risk, i.e. presence of diagnosed CVD, hypertension or diabetes. The other remaining 37 study groups consisted of healthy subjects. The time between repeated FMD measurements ranged from 25 minutes to 9 months. Mean baseline brachial diameter was 3.9 mm (range, 3.5 to 4.7 mm) and mean baseline FMD (i.e. this first of the two repeated measurements) was 7.1% (range, 1.8 to 19.9%). The FMD TEE ranged from 0.33 to 4.83% across study groups, with a mean value of 1.4%. The level of experience for each centre at the time of publication of the reproducibility study in question varied widely (number of previously published studies on FMD ranging from 0 to 71, median of 3).

Methodology-related factors *versus* variation in FMD

There was considerable variation in the methodological factors between studies. Adherence scores ranged from 2.4 to 9.2, with a mean of 5.3 (out of a maximum of 10). The adherence score was inversely correlated with log FMD TEE (adjusted R^2=0.36, P<0.01, Figure 2).

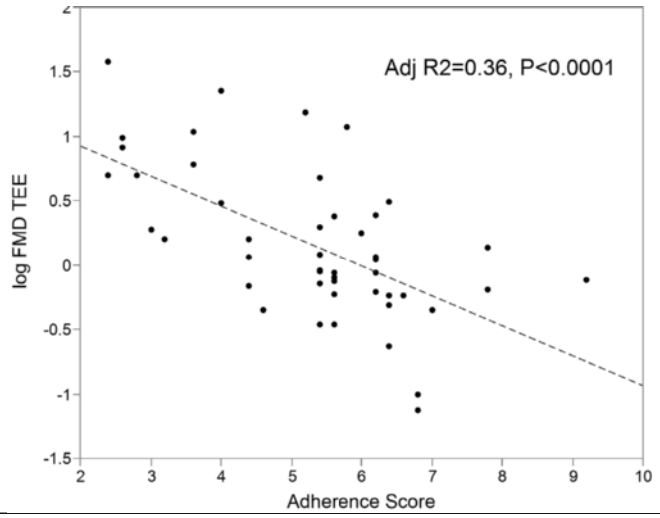

Figure 2. Linear correlation between the Typical Error of the Flow Mediated Dilation Estimate (FMD TEE) and adherence to expert guidelines (Adherence Score) in 27 studies (involving 48 study groups) of FMD reproducibility.

To explore the impact of the different aspects of the adherence score on the FMD TEE, we compared the FMD TEE between adherence (Yes *vs* No) to various methodological variables. Statistically significant differences in FMD TEE were found for use of the true peak diameter to calculate %FMD, continuous brachial artery diameter measurement over the cardiac cycle, use of automated edge detection software and smoking cessation prior to measurements (Table 2).

Table 2. Relationship of individual components of the adherence score with FMD TEE

Adherence Score Characteristic	**Median (IQR) %FMD TEE**						
Subject preparation	**n**		**No**	**n**		**Yes**	**p**
Fasting state (>6h)	21	1.08	(0.83-2.10)	27	0.96	(0.80-1.47)	0.38
No smoking/tobacco consumption prior to measurement (>6h)	22	0.89	(0.73-1.22)	26	1.30	(0.89-2.55)	**<0.01**
No habitual exercise prior to measurement (>48h)	31	1.22	(0.89-1.97)	17	0.87	(0.72-1.36)	0.07
No food/beverages that contain alcohol and/or caffeine for >12 h	31	1.06	(0.79-1.97)	17	1.04	(0.82-1.40)	0.6
No polyphenol-rich food/beverages (cocoa, tea, fruit juices) for >18 h	45	1.04	(0.79-1.63)	3	1.15	(0.83-2.91)	0.6
No vitamins for at least 72h	44	1.05	(0.80-1.63)	4	0.99	(0.68-2.47)	0.8
Vasoactive medications withheld/noted on the morning of the study	26	1.01	(0.79-1.98)	22	1.06	(0.84-1.51)	0.8
Supine position; ≥15 min rest in a quiet, temperature controlled room	30	1.01	(0.80-1.72)	18	1.10	(0.82-1.60)	1.0
Repeated measurements standardised to timing of the menstrual cycle	36	1.01	(0.79-1.89)	12	1.06	(0.84-1.59)	0.7
Repeated measurements done in fixed time windows (same time of day)	7	1.22	(0.94-1.47)	41	0.96	(0.79-1.80)	0.5
Image acquisition							
Diameter measurements recorded continuously over the cardiac cycle	35	1.22	(0.85-2.01)	13	0.88	(0.75-0.95)	**<0.01**
Diameter measurements obtained during end diastole only	15	0.89	(0.79-1.15)	33	1.22	(0.83-2.01)	0.06
Simultaneous acquisition of pulse-wave Doppler velocity signal for quantification of shear stimulus	20	1.40	(0.86-2.15)	28	0.94	(0.79-1.21)	0.05
Image analysis							
Analysis using automated edge detection and wall tracking software	13	2.19	(1.47-2.87)	35	0.91	(0.79-1.22)	**<0.01**
FMD calculation point (true peak diameter)	17	1.63	(0.94-2.76)	31	0.91	(0.79-1.28)	**<0.01**
Lab data							
Use of experienced sonographers reported	20	1.09	(0.73-2.38)	28	1.05	(0.82-1.47)	0.7
Same sonographers paired to same subjects for repeated measurements	8	1.10	(0.86-1.44)	40	1.01	(0.79-1.89)	0.8

Subject-related factors *versus* variation in FMD

For the remaining methodology related factors and subject characteristics, there were weak, but statistically significant correlations of log FMD TEE with age (adjusted R^2= -0.18, P<0.01) and with baseline FMD (adjusted R^2 0.11, P=0.013). In addition, FMD TEE was significantly smaller in the subgroup of studies that applied a stereotactic probe holder, and in studies performed by groups with more experience according to number of earlier publications on FMD. The %FMD TEE of studies above and below the median duration between repeated measurements (7 days) was not significantly different (Table 3), and there was no correlation between %FMD TEE and the time between repeated measurements (adjusted R^2= -0.02, P<0.75).

Table 3. Relationship of subject- and methodology-related characteristics with FMD TEE

Continuous variables	**Adjusted Pearson R^2**	**P-values**
Age (years)	-0.18	**<0.01**
Baseline FMD (%)‡	0.11	**0.01**
Baseline diameter (mm)	-0.02	0.15
Number of subjects (n)	-0.001	0.33

Categorical variables	**Median (IQR) %FMD TTE**						
	n		**No**	**n**		**Yes**	**P-values**
CVD risk	37	1.15	(0.79-2.01)	11	0.94	(0.85-1.07)	0.31
Distal occlusion cuff placement	5	2.01	(0.91-2.6)	43	1.04	(0.79-1.47)	0.17
Stereotactic probe holder	18	1.82	(1.02-2.85)	30	0.92	(0.73-1.22)	**<0.01**
Experienced centre*	23	1.32	(0.88-2.5)	25	0.91	(0.80-1.19)	**0.01**
Time between repeated measurements above median†	18	0.94	(0.81-1.72)	30	1.06	(0.71-1.61)	0.77

‡ Baseline FMD refers to the first of the two repeated measurements

*Centre experience was defined as the number of previous studies on FMD published by the principle author of each included study. The effect of centre experience was examined by comparing the %FMD TEE of studies below (No) and above (yes) the median number of previously published FMD studies.

†The effect of the time duration between studies was examined by comparing the %FMD TEE of studies below (no) and above (yes) the median duration of 7 days.

We constructed a stepwise multivariate regression model with log FMD TEE as the dependent variable and all factors that significantly influenced FMD TEE based on the individual analyses (adherence score, age, baseline FMD, probe holder and previous experience). The stepwise multivariate regression model predicted 51% of the variability in log FMD TEE. Adherence score (β= -0.16), age (β = -0.01) and probe holder (β = -0.19) remained as statistically significant (P<0.05) predictors in the model (Table 4).

Table 4. Relation of the adherence score, subject- and methodological factors with the reproducibility of the FMD measurement

Stepwise Regression Analysis (model Adj R^2=0.51)			
Variable	**β**	**95% CI**	**P-value**
Adherence Score (unit)	-0.16	-0.24; -0.07	**<0.01**
Age (year)	-0.01	-0.02; -0.001	**0.03**
Stereotactic probe holder (yes)	-0.19	-0.06; -0.33	**<0.01**

The regression coefficient β represents the increase in the log FMD TEE per unit increase in each factor. Baseline FMD and Centre experience did not remain in the model

Discussion

In the recent years the measurement of FMD in the brachial artery has been associated with predictive capacity for future CVD events. Despite this, and the relatively straightforward and non-invasive approach to its measurement, the clinical use of the FMD is hampered by its sensitivity to variations in methodology.

Our systematic analysis of previous studies that explored FMD reproducibility provides us with a number of novel observations. First, we found considerable variation in the methodology applied to measure FMD and consequently, differences between studies in the adherence to current expert consensus guidelines. Secondly, these data show a robust inverse association between adherence to the guidelines and FMD reproducibility, with higher adherence to guidelines being related to smaller variation in FMD. Thirdly, we identified methodological factors that were associated with smaller variation in FMD. Specifically, the use of automated edge detection software, continuous measurement of brachial artery diameter across the cardiac cycle, calculating %FMD by means of the true peak diameter and use of a stereotactic probe holder were related to a better reproducibility. Taken together, our study provides strong scientific data that highlight the importance of rigorous application of standardized contemporary methodology to reduce measurement error of FMD and, consequently, improve its use in (pre)clinical studies.

To our knowledge, no previous study has explored the (relative) importance of adherence to expert consensus guidelines for measures of vascular health, including frequently used techniques like intima-media thickness, pulse wave velocity, and finger photoplethysmography. Taking all studies on the reproducibility of the FMD together, involving 1,537 subjects, we found a TEE of 1.4% based on an average FMD of 7.1%. This indicates an overall good-to-acceptable reproducibility of the FMD. However, significant variation was observed between studies, with adherence to the expert consensus guidelines representing an important determinant of this variation. Our data suggests that roughly 36% of the variation in FMD

reproducibility can be explained through adherence to the guidelines alone. The presence of a linear relation between adherence to the guidelines and variation of the FMD suggests that measurement error would be further reduced with stricter adherence to the guidelines. Our data also indicate that even with full adherence to current expert consensus guidelines, some level of measurement error remains present. Nonetheless, a significant amount of variation in the FMD can be prevented by strong adherence to guidelines.

Our analysis provides further insight into methodological factors that determine within-person error of the FMD measurement. For example, we found that taking the true peak artery diameter (rather than a fixed time point), continuous diameter measurement and automated edge-detection contribute to minimizing measurement error. The importance of these methodological factors have already been acknowledged in previous work. For example, Black *et al.* found that the peak diameter following cuff release differs between young and older subjects.[7] Consequently, calculating the FMD% at an arbitrary time point (e.g. 60 seconds) may lead to misleading conclusions compared to an approach in which diameter of the brachial artery is recorded continuously, allowing for the detection of the true peak dilation. Furthermore, previous work demonstrated that the adoption of edge-detection software to perform observer-independent analysis leads to smaller variation compared to the application of manual calipers, the latter being highly prone to measurement bias.[36, 41, 42] Whilst these studies highlight the importance of considering these factors for valid use of FMD, the present study also highlights the importance of considering these factors to lower variation. Therefore, our study provides an additional rationale to perform continuous assessment of the diameter and the adoption of edge-detection software when performing valid and reproducible assessments of the FMD.

Another important observation in our study was that previous experience of a laboratory with the FMD resulted in a smaller variation. A potential explanation for this finding is that experienced laboratories are more likely to demonstrate better adherence to the expert consensus guidelines. Indeed, when all factors were included in the final regression analysis (including adherence to the guidelines), previous experience of a laboratory with FMD did not emerge as an independent predictor of FMD reproducibility. Another factor that contributed to a smaller variation of the FMD was the use of a probe holder. The use of such devices is largely dependent on the personal preference of the laboratory and the effect on measurement reproducibility is a complex topic, since highly skilled operators with years of experience are able to conduct FMD measurements with exceptional reproducibility, regardless of the use of a probe holder.[19] One may speculate that sonographers' learning curves will likely differ depending on whether a probe holder is used or not and also depending on the design and construction of the probe holder itself. Therefore, despite the significant inverse association in our analysis, it remains difficult to ascertain whether use of a probe

holder leads to a smaller variation in FMD *per se*. Further studies are needed to confirm the importance of using a probe holder to reduce variability of the FMD.

Of the subject-related factors (age, diameter and baseline FMD), only age contributed independently to the variation in FMD. Notably, higher age of subjects was associated with a smaller variation in FMD. Older age is typically associated with a lower FMD,[43, 44] which may contribute to a smaller (biological) variation and/or less ability to change in response to hemodynamic stimuli, consequently leading to a smaller measurement error. However, at least a previous work suggests the presence of larger variability for measurements of vascular health in clinical groups. For example, Craiem *et al.* found that subjects with CVD, despite comparable baseline FMD% values, demonstrate a larger coefficient of variation compared to healthy controls.[22] Our data suggest that the reproducibility of the FMD may differ between (clinical) groups.

Interestingly, the time between repeated measurements did not significantly affect FMD reproducibility in our analyses. This might seem counter-intuitive, as poorer reproducibility is expected as the time between repeated measurements increases. Indeed, a recent study specifically designed to determine FMD reproducibility over short (48 hours), medium (3 months) and long (9 months) time frames did find poorer reproducibility at 9 months between repeated measurements.[15] Reproducibility was comparable for the shorter time periods however, which is in agreement with a recent Italian multicentre study which found no differences in FMD reproducibility up to 30 days between measurements.[16] It should be noted that there was a large heterogeneity in time between measurements in the included study groups, with the majority ranging between one and 15 days (n = 32) and some up to 30 (n=11), 90 (n = 4) and 270 days (n = 1). Excluding these last 16 studies from the analyses did not appreciably change our findings our findings however (data not shown).

Limitations. An obvious limitation of our systematic review is that the degree of adherence to expert consensus guidelines was assessed from information as provided in the papers. If a methodological description omitted one or more of the 19 different scoring factors, no points were assigned for those factors. As a consequence some studies with sparse methodological descriptions received lower scores. Poor methodological reporting might therefore have confounded our outcomes. It should also be acknowledged that our estimation of the experience of a laboratory with FMD measurements does not necessarily reflect the experience of an individual sonographer. However, a laboratory more experienced in performing FMD measurements will generally require a level of skill and training for their sonographers that will meet at least the standard of their previous work. This highlights the importance of the level of experience in performing studies with FMD as an outcome variable. Another limitation is that our analysis on the relative importance of individual subject- and/or

methodology-related factors could only be based on a between-study comparison of factors contributing to the reproducibility of the FMD. Various other factors may have influenced this analysis. Therefore, future studies are necessary to further explore the importance of (some of) the methodology-related factors, including the effects of factors which we could not examine with the current dataset such as the observer/analyst, the time of cuff occlusion and changes in baseline brachial artery diameter.

In conclusion, this systematic review shows that adherence to current expert consensus guidelines significantly reduces measurement error when assessing brachial artery FMD in humans. Moreover, when adopting the guidelines, we found that the use of contemporary techniques (i.e. continuous diameter recording, edge-detection and wall-tracking software and under some circumstances the use of a probe holder) is crucial to improve reproducibility of the FMD measurement. Considering these factors will importantly decrease measurement error of the FMD and, consequently, decrease chances for type II errors in studies that rely on FMD as their primary outcome parameter. In other words, ignoring current expert-consensus guidelines causes significant variability of the FMD and, consequently, may lead to spurious conclusions. This study delivers important insight that should be taken into account when developing future updates to expert-consensus guidelines.

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Chapter 3b

Data in brief; assessing the perceived quality of brachial artery Flow Mediated Dilation studies for inclusion in meta-analyses and systematic reviews: description of data employed in the development of a scoring tool based on currently accepted guidelines.

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Data in Brief. 2016

Abstract

Brachial artery flow mediated dilation (FMD) is widely used as a non-invasive measure of endothelial function. Adherence to expert consensus guidelines on FMD measurement has been found to be of vital importance to obtain reproducible data. This article lists the literature data which was considered in the development of a tool to aid in the objective judgement of the extent to which published studies adhered to expert guidelines for FMD measurement. Application of this tool in a systematic review of FMD studies (http://dx.doi.org/10.1016/j.atherosclerosis.2016.03.011)1 indicated that adherence to expert consensus guidelines is strongly correlated to the reproducibility of FMD data.

Specifications Table

Subject area	*Medicine*
More specific subject area	*Vascular Physiology*
Type of data	*Table*
How data was acquired	*Systematic literature survey and expert consensus*
Data format	*Processed*
Experimental factors	*Methodological parameters related to valid measurement FMD*
Experimental features	*Assessment tool based on 33 studies pertaining to the most appropriate methods to assess FMD in humans identified from literature and expert guidelines for FMD measurement*
Data source location	*Nijmegen, The Netherlands*
Data accessibility	*Data is within this article*

Value of the data

- The literature data provided here establishes an evidence base and a physiological background rationale for the individual components included in the Adherence Score, aiding in the improvement of the practical guidance and technical approaches to FMD measurement and analysis.
- This "Adherence Score" which ranges between 0 (i.e. no adherence) and 10 (i.e. full adherence) can conceivably be employed to evaluate the perceived quality of studies reporting FMD data, with a higher outcome of this measure being strongly related to better reproducibility of the FMD data.
- This tool may prove useful additional information when pooling, contrasting and comparing different studies, e.g. for the purpose of meta-analyses or systematic reviews.

Data

A tool to enable objective assessment of the level adherence to the FMD guidelines was developed. Table 1 presents the 19 different factors that make up the *"Adherence Score"* tool along with citations to the literature data which justify the inclusion of each factor in question.

Table 1. Scoring tool based on currently accepted guidelines for the assessment of the perceived quality of FMD studies

Characteristic	Score	Ref.
Subject preparation		
Fasting state (>6h)	Yes 0.2; No 0	1-3
No smoking or any tobacco consumption prior to measurement (>6h)	Yes 0.2; No 0	4-6
No habitual exercise prior to measurement (>48h)	Yes 0.2; No 0	7-9
No food/beverages that contain alcohol and/or caffeine for >12 h	Yes 0.2; No 0	10, 11
No food/beverages that are rich in polyphenols (cocoa, tea, fruit juices) for >18 h	Yes 0.2; No 0	12
No vitamins for at least 72h	Yes 0.2; No 0	13-15
Vasoactive medications withheld on the morning of the study if possible for single measurements; Careful noting of the use and timing of any drugs in the case repeated measurements	Yes 0.2; No 0	16, 17
Supine position; Rest for at least 15 min prior to measurements in a quiet, temperature controlled room	Yes 0.2; No 0	18-20
In female subjects, repetitive measurement should be made at the same time of the menstrual cycle (ideally on days 1–7)	Yes 0.2; No 0	21, 22
Repeated measurements done in fixed time windows (same time of day)	Yes 0.2; No 0	23-25
Image acquisition		
Diameter measurements recorded continuously + over the heart cycle OR; Diameter measurements obtained during end diastole only	Yes 2; No 0 Yes 1; No 0	26, 27
Simultaneous acquisition of pulse-wave Doppler velocity signal for quantification of shear stimulus	Yes & insonation angle ≤60° 2; Yes & insonation angle >60°/not reported 1; No 0	28-30
Image analysis		
Analysis using automated edge detection and wall tracking software	Yes & continuous (i.e. time bins of ≤5 seconds) 2; Yes & fixed time points 1; No 0	31-33
Laboratory information		
Use of experienced sonographers reported	Yes 1; No 0	
Same sonographers paired to same subjects for repeated measurements	Yes 1; No 0	

Experimental Design, Materials and Methods

Based on previous expert-consensus guidelines,[34] we devised a scoring system reliant on the reporting of 19 different methodological factors related to FMD measurement. These factors were identified after critical review and appraisal of published physiological studies pertaining to the most appropriate methods to assess FMD in humans. Values were assigned to each component proportional to its perceived importance for valid assessment of the FMD. This was done through expert consensus discussion within the Working Group (AG, LG and DHJT). The *"Adherence Score"* that any given study can be assigned ranges from 0 to 10 points depending on how many of the 19 different factors that are reported or referred to in the text of the paper in question.

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Chapter 3c

Letter to the Editor; Reply to Sabour *et al.* regarding 'Adherence to guidelines strongly improves reproducibility of brachial artery flow-mediated dilation'.

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Atherosclerosis. 2016

To the editor

We thank Dr. Sabour[1] for his interest in our recent publication.[2] In our study we conducted a systematic review of studies reporting on the reproducibility of brachial artery flow-mediated dilation (FMD). Subsequently, we determined the relation between the adherence of these studies to current expert guidelines for FMD measurement and its reproducibility of the FMD.

Dr. Sabour raises important points around analysis of reliability. More specifically, Dr. Sabour strongly supports the use of the intraclass correlation coefficient (ICC) when examining reliability. It should be noted, however, that our paper does not necessarily relate to this work on *reliability*. In fact, we have not performed any specific analysis on reliability and/or reproducibility. Adopting the ICC approach in our study would have required us to have access to the original individual data of the studies identified in our systematic review (which we did not). We simply correlated a predefined and standardized measure of reproducibility[3] (that was calculated and presented in previous studies) to a measure of reported adherence to guidelines.

The above approach led us to conclude that studies which reported high adherence to current expert consensus guidelines and use contemporary techniques for measuring brachial artery FMD were related to lower measurement error. As such we would reiterate that ignoring current expert-consensus guidelines causes significant variability of the FMD and, consequently, may lead to spurious conclusions.

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The cold never bothered me anyway

Part 2

Carotid artery reactivity

Chapter 4

Correlation of carotid artery reactivity with cardiovascular risk factors and coronary artery vasodilator responses in asymptomatic, healthy volunteers.

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Abstract

Objectives. Carotid artery reactivity (CAR%), involving carotid artery diameter responses to a cold pressor test, is a non-invasive measure of conduit artery function in humans. This study examined: 1. the impact of age and cardiovascular risk factors on the CAR% and 2. The relationship between CAR% and coronary artery vasodilator responses to the cold pressor test.

Methods. Ultrasound was used to measure resting and peak carotid artery diameters during the cold pressor test, with CAR% being calculated as the relative change from baseline (%). We compared CAR% between young (n=50, 24±3 years) and older participants (n=44, 61±8 years), and subsequently assessed relationships between CAR% and traditional cardiovascular risk factors in 50 participants (44±21 years). Subsequently, we compared left anterior descending (LAD) artery velocity (using transthoracic Doppler) with carotid artery diameter (i.e. CAR%) during the cold pressor test (CPT, n=33, 37±17 years).

Results. A significantly larger CAR% was found in young versus older healthy participants (4.1±3.7 versus 1.8±2.6, P<0.001). Participants without cardiovascular risk factors demonstrated a higher CAR% compared to those with ≥2 risk factors (2.9±2.9 versus 0.5±2.9, P=0.019). Carotid artery diameter and LAD velocity increased during CPT (P<0.001). Carotid diameter and change in velocity correlated with LAD velocity (r=0.486 and 0.402, P<0.004 and 0.02, respectively).

Conclusion. Older age and cardiovascular risk factors are related to lower CAR%, whilst CAR% shows good correlation with coronary artery responses to the CPT. Therefore, CAR% may represent a valuable technique to assess cardiovascular risk, whilst CAR% seems to reflect coronary artery vasodilator function.

Introduction

Previous studies have explored the impact of stimulation of the sympathetic nervous system, using the cold pressor test (CPT), on coronary artery responses.[1-3] Coronary artery responses to CPT are suggested to be endothelium-dependent.[4] Whilst coronary dilation is observed in healthy volunteers, participants with CV risk or disease demonstrate attenuated dilation or even constriction during the CPT.[2, 4-8] Moreover, CPT-induced constriction of coronary arteries independently predicts future cardiovascular (CV) events.[9] Non-invasive assessment of coronary artery diameter, however, is currently technically challenging, expensive and lacks sufficient temporal resolution to assess rapid changes in diameter.

Similar to coronary arteries, CPT may dilate carotid artery in asymptomatic older participants, whereas significant constriction is present in those with coronary heart disease.[10] No previous study examined whether the magnitude of response (i.e. dilation or constriction) of the carotid artery reactivity (CAR%) to the CPT is altered by older age and/or presence of cardiovascular risk factors. Furthermore, given similarity in vascular responsiveness between coronary and carotid arteries to CPT, with opposite responses between healthy participants (i.e. dilation) *versus* patients with coronary heart disease (i.e. constriction),[9, 10] one may question whether a correlation exists between coronary and carotid artery responses to the CPT, such as described previously for other measures of peripheral vascular function.[11-16] This would provide the first study to assess whether CAR% directly relates to coronary artery vascular function.

This study aims to better understand the potential clinical relevance of CAR% as a putative marker of cardiovascular risk and surrogate for coronary artery function. First, we examined the hypothesis that older age and increasing number of traditional cardiovascular risk factors (e.g. blood pressure, cholesterol, hypertension, diabetes, and smoking) are associated with a smaller CAR% in healthy, asymptomatic participants. Secondly, we explored the relation between coronary artery and carotid artery responses to the CPT in healthy, asymptomatic participants. This work will provide important information to determine if the carotid and coronary arteries exhibit similar functional responses in the presence of cardiovascular risk factors and disease.

Methods

Participants

We recruited 94 healthy participants without clinical presentation of atherosclerosis. Exclusion criteria were a history of cardiovascular disease (i.e. angina, myocardial infarction,

and heart failure), presence of Raynaud's phenomenon, scleroderma, chronic pain and/or open wounds on the upper extremities. Written informed consent was obtained from all participants prior to participation. Ethical approval was obtained from local Ethics committee (Aim 1: Radboud university medical centre, Aim 2: Liverpool John Moores University), in accordance with the latest revision of the Declaration of Helsinki.

Experimental design

All participants (n=94) reported to our laboratory for a single visit. Participants were asked to abstain from strenuous exercise for 24 hours, fast for ≥6 hours, and to abstain from dietary products known to alter endothelial function for ≥18 hours prior to the testing sessions (i.e. caffeine, vitamin C) according to guidelines to assess peripheral vascular function.[17] Upon arrival, weight (kg) and height (cm) were measured and participants rested in the supine position for at least 15 minutes on a comfortable bed in a temperature-controlled room. All subjects underwent the CPT, involving continuous ultrasonography measurements of the carotid artery diameter and velocity as well as haemodynamics at baseline (1-min) and during (3-min) CPT. Peak changes in diameter during CPT, presented as the relative change from baseline, represents the CAR%. To reduce measurement error, procedures were repeated after 1 h and averaged for analyses. For Aim 1 (i.e. relationship CAR% & risk factors), we divided the entire study population (n=94) into young (n=50, age range 19-30 years) and older adults (n=44, age range 50-82 years). Cardiovascular risk profile was assessed in 50 participants (Radboud university medical centre, 44±21 years), who were divided in subjects with 0, 1 or ≥2 cardiovascular risk factors. These different subgroups are presented in Figure 1.

For Aim 2 (i.e. CAR% *vs* coronary artery velocity), we studied a subgroup of 44 participants (Liverpool John Moores University), and simultaneously examined carotid artery diameter and left anterior descending coronary artery velocity responses using Doppler ultrasound during the CPT. Due to technical constraints 11 participants were excluded from analysis. This left us with 33 participants to assess the relation between CAR% and coronary artery velocity responses to the CPT (37±17 years).

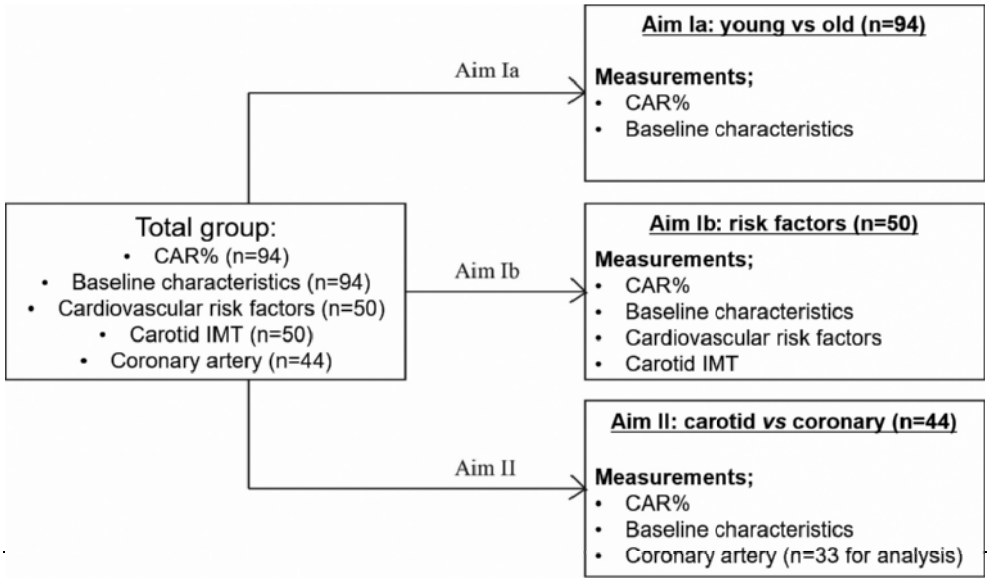

Figure 1. Flow diagram

Experimental measures

Cold pressor test. The CPT consisted of a 3-minute immersion of the left hand in a bucket of ice slush (~4.0°C). The participant was positioned supine on a comfortable bed, facilitating arm movement of the left hand into the bucket of ice slush without significant movement of the neck to enable assessment of the carotid and coronary arteries. After a 1-minute baseline period, the participant immersed the hand up to the wrist in the ice slush for 3 minutes. The participant was instructed not to speak and breathe normally (to prevent hyperventilation) when the hand was submerged into the ice slush.

Carotid artery diameter, blood flow and shear rate. Participants were positioned with the neck extended to allow assessment of the carotid artery. Left carotid artery diameter and red blood cell velocity were recorded continuously during baseline (1-minute) and CPT (3-minutes) with a 10-MHz linear array handheld probe attached to a high resolution ultrasound machine (Terason T3000, Aloka, United Kingdom). When an optimal image was found, the probe was held stable and the ultrasound parameters were set to optimise the longitudinal, B-mode image of the lumen-arterial wall interface. Continuous pulsed wave Doppler velocity assessments were also obtained and were collected at the lowest possible insonation angle (always <60°). Following a 1-minute baseline assessment of carotid artery diameter and velocity, the hand was immersed for 3-minutes with simultaneous and continuous assessment of carotid artery diameter and velocity.

Intima-media thickness. Previous studies found carotid artery intima-media thickness (IMT) to relate to cardiovascular risk and predict future cardiovascular disease.[18] To explore the relevance of studying CAR% *and* IMT, we included measurements of the IMT (mm) of the left common carotid artery. According to widely adopted recommendations, we measured the IMT approximately 2cm proximal to the bulbus. We recorded the IMT continuously for 10 seconds, in 2 different perpendicular planes (differing 90º). From the 2 measurements wall thickness was calculated. Analyses were performed with edge-detection and wall-tracking software, as described elsewhere.[19]

*Blood pressure and heart rate**.*** Before and during CPT, we continuously measured blood pressure using non-invasive photoplethysmography (Aim 1: Nexfin, BMEYE, Amsterdam, The Netherlands, Aim 2: Portapress, Finapres Medical Systems, Amsterdam, Netherlands).

Cardiovascular risk factors (Aim 1; CAR% vs Risk factors). For the subgroups of 50 participants, we performed additional assessment of cardiovascular risk factors. To examine systolic and diastolic blood pressure, we performed two assessments of blood pressure using the manual approach (sphygmomanometer, on the left arm). Hypertension was defined as systolic pressure ≥140mmHg and/or diastolic pressure ≥90mmHg.[20] We reported diagnosis of type 1 or 2 diabetes mellitus and recorded (past and current) smoking habits. We used capillary blood to assess total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol and triglycerides (35µL blood, Mission, ACON Laboratories, Inc., San Diego, USA). Elevated cholesterol levels were defined as total cholesterol >5.0 mmol/L.[21-23] Based on the presence of risk factors, these participants were subdivided in; *i.* 0 risk factors, *ii.* 1 risk factor, and *iii.* ≥2 risk factors.

Coronary artery responses (Aim 2; CAR% vs coronary artery). In a subgroup of 33 participants (37±17 years), left anterior descending (LAD) coronary artery velocity responses to the CPT were examined using transthoracic ultrasound, during simultaneous assessment of the CAR. Transthoracic assessment was performed by a highly experienced sonographer using a Vivid Q (GE Medical, Horten, Norway), with a 4 MHz phased array transducer. To this end, participants assumed a slightly left lateral position to allow access and measurement of the proximal end of the LAD from a modified parasternal window. When the vessel was detected (using colour flow mapping), the Doppler sample volume was positioned in the vessel, to allow for real-time velocity assessment during the cardiac cycle. Acquisition of the coronary velocity was obtained at baseline and during CPT.[24]

Data analysis

Carotid artery diameter, velocity, blood flow and shear rate. CAR% responses were assessed for both diameter and blood flow. Analysis of the carotid artery diameter was performed using

custom-designed edge-detection and wall-tracking software, which is largely independent of investigator bias, by a single blinded investigator.[25] Details of this technique can be found elsewhere.[26] Baseline diameter, velocity, shear rate,[25] and blood flow were calculated as the mean of data acquired across the 1 minute preceding the CPT test. After submersion of the hand in ice slush, data were calculated as the mean value for 10-second intervals, involving 8-10 full cardiac cycles. Based on this data we calculated the peak diameter change (i.e. the 10-second bin with the highest value, CAR%) and area-under-the-curve for the diameter change during CPT (CAR_{AUC}). The peak diameter change can refer to a maximum constriction *or* dilation. The direction of this change was determined by a positive (i.e. dilation) or negative (i.e. constriction) CAR_{AUC}. In keeping with previous work, we also calculated the diameter change at 90 seconds (CAR_{90}).[17] Reproducibility (coefficient of variation, CV) of diameter responses to CPT was assessed with a 1- and 24-hour interval. Within-day CV for baseline and peak diameters was 2.2 and 2.6%, whilst day-to-day CV were 2.3% and 2.7%. Furthermore, the CAR% (i.e. maximum change in diameter) showed a within-day reproducibility of 2.6% and between-day reproducibility of 2.8%.

Blood pressure and heart rate. Analyses included baseline and peak mean arterial pressure (MAP, mmHg), and baseline and peak heart rate (HR, beats per minute). Analyses were performed in labchart (LabChart 7, ADInstruments, Colorado Springs, USA) and/or excel. Both MAP and HR were averaged per 30 second bins for analyses. All values were averaged over the 2 CPTs.

Coronary artery responses. All images were exported to DVD in raw format, for offline analyses. The coronary blood velocity was analysed using commercially available software (EchoPAC Version 7.0; GE Medical, Horten, Norway). Measurements were performed at both baseline and during CPT and included peak systolic (S), peak diastolic (D) velocity and the velocity time integral (VTI).

Statistical analysis

All data were presented as mean ± SD unless stated otherwise. Statistical analysis was done using IBM SPSS Statistics 20.0 (IBM SPSS, IBM Corp., Armonk, NY, USA). For Aim 1, we examined differences between young and older groups using an independent Students' *t*-tests (when data were normally distributed, following Kolmogorov-Smirnov tests of normality) or Mann-Whitney U tests (when data was not normally distributed). Effects of CPT differences between the groups (young *vs* older, and 0 *vs* 1 *vs* ≥2 risk factors) and time (baseline *vs* CPT) was assessed by 2-way repeated measures ANOVAs. Subsequently, 50 individuals with assessment of traditional cardiovascular risk factors were categorised into presence of 0, 1 or ≥2 cardiovascular risk factors. A one-way ANOVA (data normally distributed) or Kruskal-Wallis (data not normally distributed) was adopted to examine differences in our primary outcome

parameters between groups. A Pearson's correlation was adopted to assess the relation between CAR% (i.e. carotid artery function) and carotid artery intima-media thickness and diameter (i.e. carotid artery structure). For Aim 2, we first examined the change in carotid artery diameter and LAD velocity in response to CPT using a paired Student's *t*-tests. Pearsons' correlation coefficient was used to explore the relation between the change in carotid artery diameter (i.e. CAR%) and change in coronary artery velocity (i.e. VTI).

Results

In healthy young subjects, CPT caused a gradual increase in carotid artery diameter that peaked around 90 seconds and, subsequently, returned towards baseline (Figure 2A). Carotid artery velocity and blood flow showed a gradual (~15%), but significant increase across the 3-minutes of the CPT-response (Figure 2B-C). Interestingly, shear rate remained around baseline levels until 90/100 seconds, after which it showed a marginal (~10%) increase (Figure 2D).

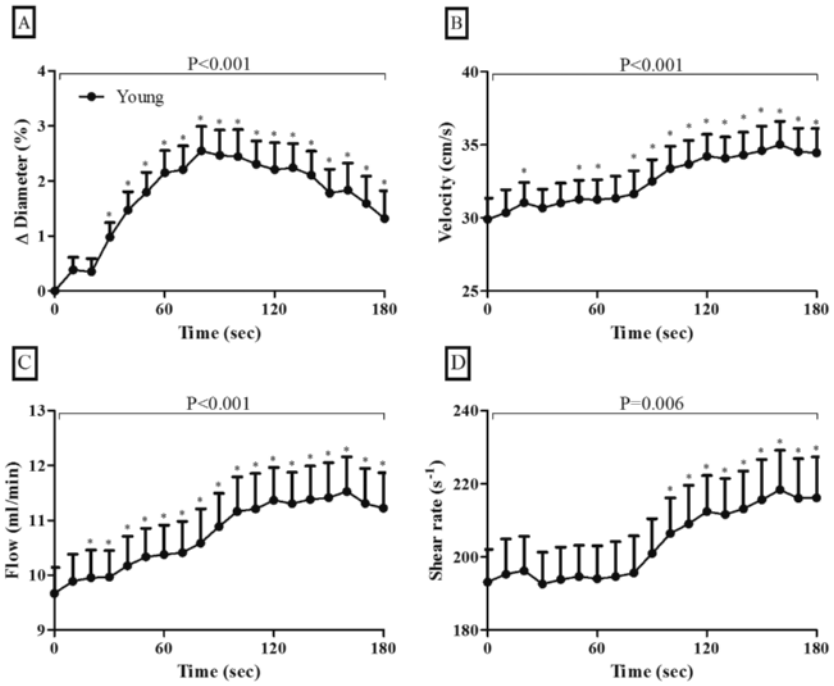

Figure 2. The time course adaptations.
The time course presented during the cold pressor test in a young healthy subpopulation (n=25). A; diameter over time (cm), B; flow velocity over time (m/sec), and C; blood flow (ml/min) and D; shear over time (s-1). Error bars represent SEM.

Aim 1: CAR% *versus* cardiovascular risk factors

Young and older participants. Older participants demonstrated higher weight and BMI, but no differences in height (Table 1). Systolic and diastolic blood pressure were higher in older compared to young participants (Table 1). Mean arterial pressure was lower in young compared to the older group, whilst heart rate was not different between groups (Table 2). Carotid artery diameter was larger in the older group than in young participants, whilst carotid artery shear rate was higher in the young group (Table 2). CPT induced a significant increase in heart rate and mean arterial pressure in both groups, with older participants demonstrating a larger increase in heart rate and a larger increase in mean arterial pressure (Table 2). Both groups demonstrated a significant increase in carotid artery diameter in response to the CPT (Table 2). The diameter response during the CPT was significantly larger in young compared to older humans when data were presented as the peak diameter change (i.e. CAR%), area-under-the-curve across the 3-minute CPT (i.e. CAR_{AUC}) and diameter change at 90-seconds (i.e. CAR_{90}) (Table 2, Figure 3A).

Table 1. Subject characteristics.

	Young	**Older**	**P-value**
Sex (% male)	56%	64%	0.452
Age (years)	24±3	61±8	*<0.001*
Weight (kg)	69±12	77±13	*0.003*
Height (m)	174±8	172±8	0.100*
Body Mass Index (kg/m^2)	23±3	26±4	*<0.001**
Systolic blood pressure (mmHg)	118±9	134±19	*<0.001**
Diastolic blood pressure (mmHg)	68±8	78±7	*<0.001*

Subject characteristics for the comparison between young (19-30 years, n=50) and older (>50 years, n=44) participants. P-value refers to an unpaired Student's t-test or *Mann-Whitney U test for the comparison between young and older participants.

Table 2. Carotid artery and hemodynamic baseline characteristics.

	Young		Older		2-way ANOVA		
	Rest	*CPT*	*Rest*	*CPT*	*group*	*CPT*	*Group*CPT*
MAP (mmHg)	85±13	95±14	102±15	114±18	*<0.001*	*<0.001*	0.063
HR (bpm)	64±12	65±11	59±9	64±10	0.073	*<0.001*	*0.006*
Diameter (mm)	6.3±0.5	6.5±0.5	7.1±0.7	7.2±0.8	*<0.001*	*<0.001*	*<0.001*
Shear rate (1/s)	184±43	186±43	143±42	141±47	*<0.001*	0.905	0.318
Flow (ml/min)	9.2±2.3	10.1±2.6	10.2±2.8	10.3±3.5	0.286	*0.001*	*0.019*
Carotid artery reactivity (CAR)							
Diameter change (CAR%)	4.1±3.7		1.8±2.6		*<0.001**		
Diameter change$_{AUC}$ (CAR$_{AUC}$)	2.7±2.3		1.0±1.3		*<0.001*		
Diameter change$_{90}$ (CAR$_{90}$)	3.5±2.8		1.4±1.6		*<0.001*		

Carotid artery and hemodynamic baseline characteristics (averaged across a 1-minute period) and change during the cold pressor test (averaged across the 3-minute cold pressor test) in young (19-30 years, n=50) and older (>50 years, n=44) participants. P-values refer to 2-way repeated measures ANOVA's, for within participant comparison (CPT), between group comparison (group), and the interaction Group*CPT. *Refers to Mann-Whitney U test.

Cardiovascular risk factors. Cholesterol and LDL levels were highest in those with 1 RF compared to 0 or ≥2 RF, whilst no differences between groups were found for any of the other parameters (Table 3). We found a significantly different CAR%, CAR$_{AUC}$ and CAR$_{90}$ across the 3 groups (Figure 3B, Table 3), with a smaller carotid artery dilation observed in the presence of more cardiovascular risk factors. Specifically, we found that participants with ≥2 risk factors showed a smaller dilation compared to those without risk factors (Table 3). In line with the CAR%, carotid artery diameter, IMT, and IMT ratio (i.e. intima-media thickness/baseline diameter) were higher in participants with more risk factors (Table 3). However, no significant correlation was found between CAR% and carotid artery baseline diameter (r= -0.16, *P*=0.274), IMT (r= -0.09, *P*=0.524), or IMT ratio (r= -0.06, *P*=0.678).

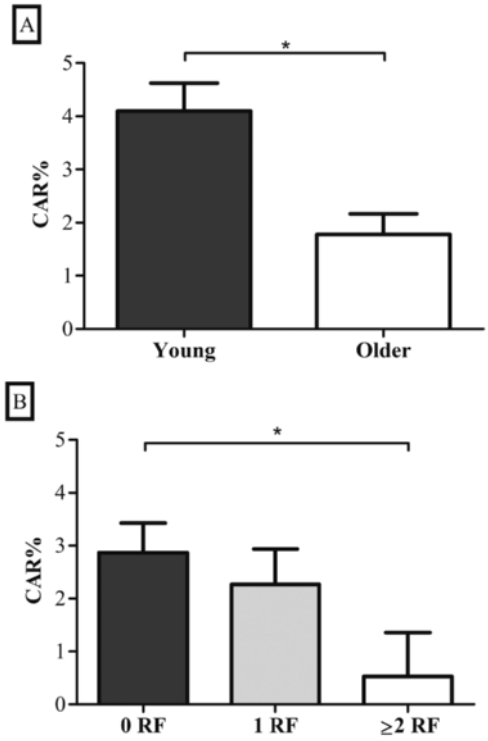

Figure 3. CAR and risk factors

Carotid artery reactivity (CAR%, presented as maximal change from baseline) in a cohort of healthy, asymptomatic subjects that were divided based on age (A: 50 young (black bar) versus 44 older humans (white bar)) and presence of cardiovascular risk factors (B: 0 risk factors (black bar, n=27), 1 risk factor (grey bar, n=11), and ≥2 risk factors (white bar, n=12)). Error bars represent SE. Statistical analysis (unpaired Students' t-test (A) and ANOVA (B)) revealed significant differences in CAR% between groups.

Table 3. CAR and risk factors.

		0 risk factors (N=27)	1 risk factor (N=11)	≥2 risk factors (N=12)	P-value
Sex (% male)		52%	55%	42%	0.794
Hypertension (%)		-	9%	17%	0.115
Diabetes (%)		-	-	8%	0.312
Smoking (%)	Current	-	9%	17%	0.139
	No	89%	64%	58%	
	History	11%	27%	25%	
Cholesterol (mmol/L)		4.25±0.7	6.17±1.4	5.5±1.3	*>0.001†*
HDL (mmol/L)		1.39±0.3	1.30±0.4	1.24±0.2	0.408
LDL (mmol/L)		2.59±0.7	4.0±1.5	3.4±1.4	*0.025*
Triglycerides (mmol/L)		1.3±1.0	2.1±1.3	1.9±1.1	0.196
Baseline diameter (cm)		0.64±0.06	0.70±0.04*	0.74±0.08*	*>0.001*
Intima-media thickness (mm)		0.60±0.2	0.75±0.1*	0.82±0.1*	*0.001*
IMT ratio		0.09±0.02	0.11±0.02	0.11±0.02*	*0.036*
Carotid artery reactivity (CAR)					
CAR%		2.9±2.9	2.3±2.2	0.5±2.9*	0.060
CAR_{AUC}		1.9±1.6	1.1±1.2	0.5±1.5*	*0.034*
CAR_{90}		2.5±2.2	1.4±1.4	0.9±1.7*	*0.037*

Carotid artery reactivity (CAR%, presented as maximal change from baseline) in a cohort of healthy, asymptomatic subjects categorised by the presence of cardiovascular risk: 1. 0 risk factors (n=27), 2. 1 risk factor (n=11), and 3. ≥2 risk factors (n=12).*Post-hoc significantly different from group 1.†Refers to Kruskall-Wallis test.HDL; High density lipoprotein, LDL; Low density lipoprotein.

Aim 2: CAR% *versus* coronary artery

The CPT caused a significant increase in heart rate, mean arterial pressure, and carotid artery flow, velocity and shear rate (Table 4). A significant increase in carotid artery diameter was found when presented as CAR%, CAR_{AUC} and CAR_{90} (Table 4). Furthermore, a significant increase in LAD velocity was found during the CPT (Table 4). We found a significant, positive correlation between the CAR% and the change in LAD velocity time integral (r=0.486, *P*<0.004, Figure 4). A significant, positive correlation was also found between changes in carotid artery velocity and flow, and the change in LAD velocity time integral (r=0.402, *P*=0.021, and r=0.368, *P*=0.035, respectively). This relation between carotid and coronary artery responses was reinforced when data were presented as CAR_{90}, but not for CAR_{AUC} (r=0.361 and 0.258, *P*=0.039 and 0.146, respectively).

Table 4. CAR and coronary responses.

	Rest	CPT	P-value
Mean arterial pressure (mmHg)	87±14	99±16	*<0.001*
Heart rate (bpm)	60±10	62±10	*0.048*
CA diameter (cm)	0.66±0.08	0.68±0.08	*<0.001*
CA shear rate (1/s)	158±46	174±43	*<0.001*
CA flow (ml/min)	9.1±2.7	10.9±3.4	*<0.001*
CA velocity (cm/s)	25.8±6.7	29.3±7.1	*<0.001*
LAD systolic velocity (cm/s)	15±3.5	18±3.4	*<0.001**
LAD diastolic velocity (cm/s)	31±7	39±9	*<0.001*
LAD velocity time integral (cm/s)	17±4	20±4	*<0.001*
Diameter change (CAR%)	4.5±3.8		
Diameter area-under-the-curve (CARAUC)	2.8±2.5		
Diameter change at 90 sec (CAR90)	3.6±2.9		
Delta VTI (cm)	2.7±2.3		

Coronary artery responses in all participants included for Aim 2 (n=33). P-value refers to a paired Student's t-test. *Refers to Wilcoxon Signed rank test.

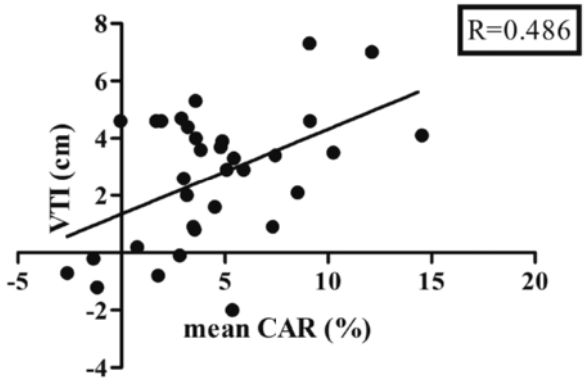

Figure 4. Correlation CAR and coronary artery

Correlation between the carotid artery diameter response (% maximum change from baseline; i.e. CAR%) and coronary left descending artery velocity response (change in the velocity time integral (VTI in cm)) during a cold pressor test in a population of healthy, asymptomatic participants (n=33). A significant, positive correlation was observed between both measurements.

Discussion

In this study we explored the relationship between age, cardiovascular risk factors and CAR% and whether carotid artery responses to CPT reflect coronary artery vascular function. We found that the CPT induces carotid artery dilation in healthy, asymptomatic young participants, with no changes in shear rate. This highlights the ability of the carotid artery to dilate in response to the CPT, a functional change that is unlikely to be related to shear-mediated responses, as the dilation response of the carotid artery preceded any change in shear. Secondly, the CAR% was significantly attenuated in healthy, asymptomatic older participants, whilst presence of traditional cardiovascular risk factors was also associated with a smaller CAR%. These findings cannot be ascribed to structural characteristics of the carotid artery diameter (i.e. diameter or intima-media thickness), given the absence of a significant correlation between CAR% and these factors. Finally, a moderate-to-strong correlation was apparent between carotid artery dilation (i.e. diameter and velocity) and coronary artery dilator (i.e. velocity) responses to the CPT. These observations provide evidence that the CAR%, most likely independent of carotid artery structural characteristics, may represent a valuable test to assess arterial function and health and that it reflects coronary artery vasomotor function.

Our study reveals the novel observation that, in a healthy, asymptomatic population, who generally demonstrate carotid artery dilation in response to the CPT, the CAR% successfully distinguishes between subjects with incremental number of risk factors. Also carotid artery IMT and diameter, both predictors for CV risk,[18] were different between groups, with a higher value for those with ≥2 traditional cardiovascular risk factors. Since we found no correlation between CAR% and carotid IMT or diameter, it is possible that CAR% provides information that is independent from that of measures of carotid artery structure (i.e. diameter and IMT). This observation provides further support that CAR% may represent relevant information on CV risk.

Ideally, a test of (peripheral) vascular function related to CV risk should also reflect vascular health of coronary vessels, since coronary arteries are prone to the development of atherosclerosis and cardiovascular events. Previous studies have explored the relationship between measures of coronary and peripheral artery vascular function.[14, 16] In line with these studies, carotid artery and coronary artery responses to the CPT show a moderate-to-strong correlation, a finding that is reinforced by earlier cross-study observations of comparable coronary and carotid artery responses to the CPT; dilation in healthy subjects or constriction in those with coronary artery disease.[2, 4, 10] The ability for marked vasomotion of the carotid artery during the CPT is different to peripheral conduit arteries that typically show negligible change in diameter.[27, 28] This further highlights the potential relevance for studying the carotid artery as a surrogate for coronary artery vascular function, since both of these conduit

vessels demonstrate similar responses to the CPT. The agreement between the coronary and carotid artery responses to the CPT somewhat contrasts with the lack of correlation between measures of carotid artery atherosclerosis (i.e. intima-media thickness) and coronary artery atherosclerosis (i.e. plaque burden).[18] Our data, nonetheless, suggest that functional, rather than structural, measures in the two vascular beds may be related.

The ability of the carotid artery to dilate (or constrict) during the CPT raises questions regarding the potential underlying mechanisms. Whilst no extant study has examined the carotid artery, several studies explored pathways contributing to coronary artery vasomotion to the CPT.[1, 2, 4, 6-9, 29] First, the diameter change to the CPT may be endothelium-dependent, since coronary artery responses to the CPT and acetylcholine (i.e. an endothelium-dependent stimulus) show similarity in vasomotion.[4, 29] To explain diameter response to the CPT, an increase in shear stress during CPT may contribute to an endothelium-dependent vasodilation.[30] However, the increase in shear rate during CPT occurred *after* occurrence of the peak diameter (Figure 2), making changes in shear an unlikely explanation for carotid artery dilation. Another possibility is that the increase in blood pressure accounts for the diameter response to CPT. Indeed, we found a relation between increase in MAP and CAR%. However, the magnitude of increase in MAP did not differ between groups, whilst an increase in MAP was also observed in those who demonstrate a decrease in CAR%. This suggests that the increase in MAP is unlikely causally linked to carotid diameter changes. This notion is further supported when examining the timing of the peak responses, since peak diameter precede peak blood pressure responses by ~30 seconds. Nonetheless, we cannot exclude the possibility that increases in blood pressure contribute (partly) to the CAR%. Alternatively, the release of catecholamines during the CPT may contribute to vasomotion of the carotid artery during CPT,[31, 32] with some work linking catecholamines (e.g. norepinephrine [NE]) to coronary artery dilation in healthy vessels or constriction in diseased arteries.[2, 29] More specifically, NE may contribute to vasodilation via endothelium-dependent release of vasodilators,[1, 33] whilst a direct impact of NE on smooth muscle cells causes vasoconstriction.[34, 35] The balance between both effects may ultimately determines the vasomotor response, which could be influenced by endothelium dysfunction. Although these mechanisms were explored in coronary arteries, comparable mechanisms may be present in the carotid artery during the CPT. Further research is required to characterize the physiology of the carotid artery responses to sympathetic stimulation using the CPT.

Clinical relevance. Previous studies adopting invasive intracoronary Doppler catheters[2, 4, 29] and quantitative angiography,[2, 4, 9, 29] have shown strong predictive capacity of coronary artery responses to sympathetic stimuli for future CV disease and/or events.[6, 7, 9] Our observation of agreement between coronary and carotid artery responses to the CPT, combined with the relation of the CAR% with age and cardiovascular risk factors, suggest the potential

utility of the CAR% test. This is further supported by the observation that the CAR% provides information that seems independent from that of structural measures of the carotid artery, i.e. diameter and intima-media thickness. The potential use is further emphasised since it is easy applicable, simple, cheap, non-invasive, and requiring a minimum of training.

Limitations. We choose to group the number of cardiovascular risk factors, rather than explore the impact of individual risk factors, on the CAR%. Examining all individual risk factors would require a markedly larger sample size to properly perform statistical analyses, whilst our aim was to explore the relation between cardiovascular risk factors and the newly introduced CAR% in asymptomatic subjects. We strongly recommend future studies to explore the impact of individual risk factors to better understand how traditional risk factors affect CAR%. Secondly, due to technical restrictions, we were unable to collect LAD diameter to correlate diameter changes between both arteries. Since changes in diameter will affect measures of velocity, we may have underestimated the true correlation between both arteries in response to the CPT. Nonetheless, the significant correlation between both vascular beds, including the significant correlation between carotid artery and coronary artery velocities, emphasises the agreement between coronary and carotid responses to the CPT.

In conclusion, in the present study we found that older age and the presence of cardiovascular risk factors is related to a lower CAR%. Therefore, CAR% may represent a valuable technique to assess cardiovascular risk, which may be used in addition to structural measures of the carotid artery (i.e. diameter and intima-media thickness). In addition, the CAR% shows a good correlation with coronary artery responses to the CPT, which suggests that the CAR% represents a surrogate for coronary artery vasomotor function.

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Chapter 5

Similarity between carotid and coronary artery responses to sympathetic stimulation and the role of alpha-1 receptors in humans.

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Abstract

Background. Carotid artery (CCA) dilation occurs in healthy subjects during cold pressor test (CPT), whilst the magnitude of dilation relates to cardiovascular risk. To further explore this phenomena and mechanism, we examined carotid artery responses to different sympathetic tests, with and without α1-receptor blockade, and assessed similarity to these responses between carotid and coronary arteries.

Methods. In randomised order, 10 healthy participants (25±3 yrs) underwent sympathetic stimulation using the CPT (3-minutes left hand immersion in ice-slush) and lower-body negative pressure (LBNP). Before and during sympathetic tests, CCA diameter and velocity (Doppler ultrasound) and left anterior descending (LAD) coronary artery velocity (echocardiography) were recorded across 3-min. Measures were repeated 90-min following selective α_1-receptor blockade via oral Prazosin (0.05mg per kg bodyweight).

Results. CPT significantly increased CCA diameter, LAD maximal velocity and velocity-time integral area-under-the-curve (all $P<0.05$). In contrast, LBNP resulted in a decrease in CCA diameter, LAD maximal velocity and velocity time integral (VTI, all $P<0.05$). Following α_1-receptor blockade, CCA and LAD velocity responses to CPT were diminished. In contrast, during LBNP (-30 mmHg), α_1-receptor blockade did not alter CCA or LAD responses. Finally, changes in CCA diameter and LAD VTI-responses to sympathetic stimulation were positively correlated ($r=0.66$, $P<0.01$).

Conclusion. We found distinct carotid artery responses to different tests of sympathetic stimulation, where α_1-receptors partly contribute to CPT-induced responses. Finally, we found agreement between carotid and coronary artery responses. These data indicate similarity between carotid and coronary responses to sympathetic tests and the role of α_1-receptors that is dependent on the nature of the sympathetic challenge.

Introduction

Activation of the sympathetic nervous system (SNS) is an important and clinically-relevant prognostic stimulus to examine artery function.[1, 2] During the cold pressor test (CPT), a potent sympathetic stimulus, the coronary arteries can result in a vasoconstrictor (via α_1-receptors) or vasodilatory response (via the α_2-, and β-receptors).[3] Vasodilator pathways prevail in healthy volunteers,[4, 5] whereas experimental studies in patients with coronary artery disease demonstrate vasoconstriction during SNS activation.[6-8] Coronary artery responses to CPT independently predict future cardiovascular events in patients at risk for cardiovascular disease,[2, 9, 10] which highlights the clinical relevance of this response. However, the invasive nature of angiography make these tests impractical for large scale clinical use. Interestingly, the carotid artery shows vasodilation during SNS activation in healthy subjects, similar to coronary artery responses. This carotid dilation is abolished or even reversed to vasoconstriction in those with (increased risk for) cardiovascular disease.[11, 12] To date, relatively little is known about the underlying mechanisms for the carotid artery reactivity to SNS activation.

Previous studies in peripheral conduit arteries have reported divergent responses to different tests of SNS-activation.[5, 13-16] To date, no previous study compared vasomotor responses of the carotid artery to distinct SNS stimuli. In line with peripheral arteries (i.e., the brachial and superficial femoral artery), we expect that distinct SNS stimuli (i.e. CPT and lower body negative pressure (LBNP)) lead to distinct carotid and coronary artery responses, as these tests mediate sympathetic activation through different pathways. More specifically, CPT evokes sympathetic activation via cold stress. The LBNP test gradually decreases central blood volume which results in progressive increases in muscle sympathetic nerve activity,[17, 18] which can directly lead to constriction of the carotid diameter.
No previous study examined the potential underlying mechanisms mediating carotid artery vasomotion during SNS activation. Work in both animal and human coronary arteries revealed a central role for α_1-receptors to mediate vasomotor responses during SNS activation.[19-21] In line with this previous work, we expect that α_1-receptors, at least in part, contribute to the carotid artery responses to CPT and LBNP. Therefore, our first aim is to examine the impact of activation of the SNS, either through the CPT (i.e. elevates SNS activity and blood pressure)[22, 23] or LBNP (i.e. elevates SNS activity, with preserved blood pressure)[24, 25] on carotid artery diameter. Our second aim was to assess the role of α_1-adrenoreceptors to these carotid artery responses by using an oral, selective α_1-adrenoreceptor blocker (i.e. Prazosin).

A recent study found good agreement between carotid and coronary responses to the CPT in healthy young and older subjects.[12] To further explore this relationship, we aimed to compare the responses between the carotid artery diameter and left anterior descending coronary

artery velocity (LAD velocity) during different SNS stimuli, with and without α_1-receptor blockade. Based on previous work,[11, 12] we anticipated that there would be similarity in the magnitude and direction of the vascular responses between both the carotid artery diameter and LAD velocity, and that these responses would be partly mediated via α_1-receptors.

Methods

Ethical approval

This study was approved by the Human Ethics Committee of the University of British Columbia and conformed to the standards set by the Declaration of Helsinki. All volunteers provided written informed consent.

Participants

We recruited 10 healthy male participants (mean age 25±3 years, height 1.78±0.1m, and weight 76±9 kg). Exclusion criteria were a history of cardiovascular disease (i.e. angina pectoris, myocardial infarction, heart failure), lung disease (i.e. COPD, lung cancer), brain disease (i.e. stroke, dementia), presence of Raynaud's phenomenon, scleroderma, chronic pain and/or open wounds on the upper extremities, obesity (body mass index >30 kg/m2), diabetes mellitus type 1 or 2, history of smoking, or elevated blood pressure (systolic >130 mmHg; diastolic >85 mmHg).

Experimental design

All participants reported to our laboratory for a single visit. They were asked to abstain from strenuous exercise for 24 hours and abstain from dietary products known to affect endothelial function for ≥18 hours prior to the testing session (i.e. vitamin C, caffeine and alcohol). Moreover, participants were asked to fast for ≥2 hours, adapted from existing guidelines to assess peripheral vascular function.26 Participants rested in the supine position for >15 minutes on a bed in a temperature-controlled room (23±1°C). Subsequently, participants underwent LBNP and two CPT, in a randomly assigned order, with 45-minutes rest between tests. All tests involved simultaneous assessment of common carotid artery (CCA) diameter and velocity (ultrasound) and left anterior descending (LAD) coronary artery velocity (echocardiography) before (across a 1-minute baseline) and during sympathetic stimulation. The protocol was repeated 90-minutes after oral administration of Prazosin (i.e. α1- adrenergic receptor antagonist that effectively blocks 80% of α1-recepter activity, 0.05mg per kg body weight).[27, 28]

Experimental measures

Common carotid artery diameter and velocity. Left carotid artery diameter and red blood cell velocity were recorded simultaneously and continuously during baseline (1-minute) and sympathetic stimuli (i.e. 3-minutes CPT, and ~18-minutes LBNP). Carotid artery image acquisition was performed using a 10-MHz multifrequency linear array handheld probe attached to a high resolution ultrasound machine (15L4, Terason T3200, Burlington, MA, USA). When an optimal image was found, 2-3 cm proximal from the bifurcation, the probe was held stable and the ultrasound parameters were set to optimise the longitudinal, B-mode image of the lumen-arterial wall interface. Continuous pulsed wave Doppler velocity assessments were also obtained and were collected at the lowest possible insonation angle (always <60º). Assessment was performed by an experienced sonographer (ACCM), whom has an hour-to-hour reproducibility (i.e. coefficient of variation) of CCA baseline diameter of 0.8% and reproducibility of 0.8% for the peak CCA diameter, in line with previous findings.[12]

Coronary artery velocity. Before and during both CPT and LBNP, the left anterior descending (LAD, cm) coronary artery velocity was examined using transthoracic ultrasound. This assessment was performed simultaneously with CCA diameter and velocity responses. All echocardiographic measurements were collected by a trained sonographer (MS) on a commercially available ultrasound system (Vivid E9; GE, Fairfield, CT) using a broadband M5S 5 MHz or a 3V 3D-array transducer. In a previous study the Cronbach's alpha reliability test revealed alpha values of 0.81 and 0.89 for both max and mean LAD velocities, respectively, suggesting good consistency between LAD velocity measurements.[29] Participants assumed a left lateral position to allow for data collection. The LAD was imaged using a modified parasternal short axis view from the fourth or fifth left intercostal space, and was assessed using pulsed-wave Doppler. The transducer was positioned such that a 2- to 3-mm segment of the LAD was imaged along the long axis, taking care to align the pulse-wave cursor with the length of the vessel. With a sample volume (2.0 mm) positioned over the color Doppler signal in the LAD, measurements of the LAD velocity were collected during the sympathetic tests.

Blood pressure and heart rate. All continuously recorded cardiovascular measurements were acquired at 200 Hz using an analog-to-digital converter (Powerlab/16SP ML 880; ADInstruments, Colorado Springs, CO, USA) interfaced with a personal computer. Before and during CPT and LBNP, systolic and diastolic blood pressure (SBP and DBP, in mmHg, respectively), stroke volume (SV, ml), rate-pressure product (RPP, HR x SBP, a reliable indicator for myocardial oxygen demand),[30] and cardiac output (CO, L/min) were continuously measured using non-invasive finger photoplethysmography (Finometer Pro, Finapres medical systems, Amsterdam, Netherlands). Heart rate (HR, beats per minute) was recorded using three-lead electrocardiography, placed in lead II configuration (Bioamp, ML132, ADInstruments, Colorado Springs, CO, USA).

Sympathetic stimuli

Cold pressor test. The cold pressor tests (CPT) consisted of a 3-minute immersion of the left hand in a bucket of ice slush (~4.0°C).[12] The participant was positioned in supine position on a tilt bed, tilted slightly to the left lateral position (~25-30°), to facilitate arm movement in the bucket of slush without significant movement of the body, and provide adequate coronary assessment. After a 1-minute baseline period, the participants hand was immersed up to the wrist in the ice-slush for 3 minutes. The participant was instructed to remain quiet during the CPT to provide for valid CCA assessment. The partial pressures of end-tidal carbon dioxide ($P_{ET}CO_2$) and oxygen ($P_{ET}O_2$) were clamped at baseline values for the entire duration of the protocol to reduce the potential impact of hyperventilation on the vascular responses, upon an end-tidal forcing approach described extensively elsewhere.[31] To reduce measurement error, CPT procedures were repeated twice and averaged for analyses.[12]

Lower body negative pressure. The participant was positioned in the supine position on a tilt bed, and strapped into a custom-made airtight, lower-body suction chamber at the level of the iliac crest.[32] The LBNP chamber was then moved from supine position into a left lateral position (~25-30°) to ensure adequate coronary imaging. The lower body negative pressure test consisted of a 5-minute baseline, followed by progressive 2-minute stages, using increments of -10 mmHg, to -80 mmHg or until pre-syncope. LBNP was terminated when *a*) pre-syncope occurred, defined by a sustained drop in systolic blood pressure <80 mmHg for more than 10 seconds,[33] or *b*) upon participants request due to the onset of subjective symptoms (e.g. feelings of dizziness, nausea, faintness). During the Prazosin condition, participants were unable to last longer than -40mmHg during the LBNP test. For reliable comparison between the control and drug condition, we chose to only include data until -30mmHg.

Data analysis

Carotid artery responses. Analyses of diameter (cm), blood flow (ml/sec), blood velocity (cm/sec) and shear (s^{-1}) were performed using custom-designed edge-detection and wall-tracking software, which is largely independent of investigator bias, as was extensively described elsewhere.[34] Baseline diameter, blood flow, blood velocity and shear were calculated as the mean of data acquired across a 1-minute baseline period.[34] For the CPT, data were calculated for 10-second intervals. LBNP data was calculated per 1-minute intervals. Subsequently, offline image analysis involves the identification of the region of interest (ROI), to allow for automated calibration on the B-mode image and velocities on the Doppler assessment.[35] A ROI is drawn around the optimal B-mode image, in which a pixel-density algorithm automatically identifies the near- and far wall. Another ROI is drawn around the Doppler waveform, which is synchronized with the B-mode diameter ROI. Ultimately, this allows for blood flow and shear rate calculations.[35] Peak diameter change was calculated relative to baseline diameter.

Coronary artery responses. All images were exported for offline analysis using commercially available software (EchoPAC Version 13.0; GE Medical, Horten, Norway). All echocardiographic values represent an average value of three cardiac cycles representing the clearest of five collected images for each experimental stage. The collected waveforms were analyzed to determine mean diastolic velocity (LADVmean, cm/sec), peak diastolic velocity (LADVmax, cm/sec), and the velocity time integral (VTI, cm).[36] Coronary flow velocity reliably reflects changes in absolute coronary blood flow,[4, 5, 37] suggesting that an increase in flow velocity reflects coronary artery dilatation. Using observer-independent software, VTI is calculated as the integral of individual velocities across the cardiac cycle. Participants in whom at least 1 image was suboptimal, were excluded prior to analyses.[29]

Blood pressure and heart rate. Analyses of systolic and diastolic pressure, heart rate, cardiac output, stroke volume, and the rate-pressure product (RPP) were performed in commercially available software (LabChart V7.1, ADInstruments, Colorado Springs, CO, USA). Measurements were averaged per 10-second bins for analyses for the CPT, and 1-minute bins for the LBNP analyses. Baseline CPT was averaged over a 3-minute period. Continuous blood pressure measurements were calibrated to automated brachial blood pressure readings during baseline (HEM-775CAN, Omron Healthcare, Bannockburn, IL, USA).

Statistical analyses

All data were presented as mean±SD unless stated otherwise. Parameters were tested for normality using a Shapiro-Wilk test. Responses of the CCA (i.e. diameter (cm), blood velocity (cm/sec), flow (ml/sec) and shear (s^{-1})) and LAD (i.e. mean velocity (cm/sec), max velocity (cm/sec) and VTI (cm) were assessed during the sympathetic stimulus with paired Students' t-tests (in case of non-parametric variables, a Wilcoxon signed-rank test was performed). Changes over time were assessed with 2-way repeated measurement ANOVA's (missing values were only imputed based on previous and consecutive measurements when available). We assessed whether CCA and LAD changes in diameter, velocity, flow and shear occurred over time (i.e. within factor 'time'), and whether this differed between conditions (i.e. between factor control *vs* Prazosin) were examined. In addition to the main effects, the 'time'*'condition'-interaction revealed whether the CCA and LAD changes across time differed between the control condition and Prazosin. This was done to assess the potential role of α-receptors in mediating CCA and LAD responses. The 2-way repeated measurement ANOVA's were performed with Sidak correction to account for multiple comparisons. Data were analysed using SPSS 20.0 software (IBM SPSS, IBM Corp., Armonk, NY, USA). Values for $p<0.05$ were assumed to be statistical significant.

Results

Carotid artery responses: different SNS stimuli.

Cold pressor test. The CPT caused a significant increase in systolic and diastolic blood pressure and the rate-pressure product (RPP), whilst no change was found in stroke volume, heart rate and cardiac output (n=9, Table 1). Although the diameter (cm) of the CCA increased significantly during CPT (P<0.001, Figure 1), CCA velocity (cm/sec), flow (ml/sec) and shear rate (s^{-1}) did not change significantly across time during CPT (P>0.05, data not shown).

Lower body negative pressure. LBNP caused a gradual, but significant increase in heart rate, a decrease in stroke volume and diastolic blood pressure, whilst systolic blood pressure and cardiac output were preserved (n=9, Table 2). LBNP caused a significant decrease in CCA diameter (cm, Figure 2), whereas no changes were found in CCA velocity (cm/sec), flow (ml/sec) and shear (s^{-1}) (data not shown).

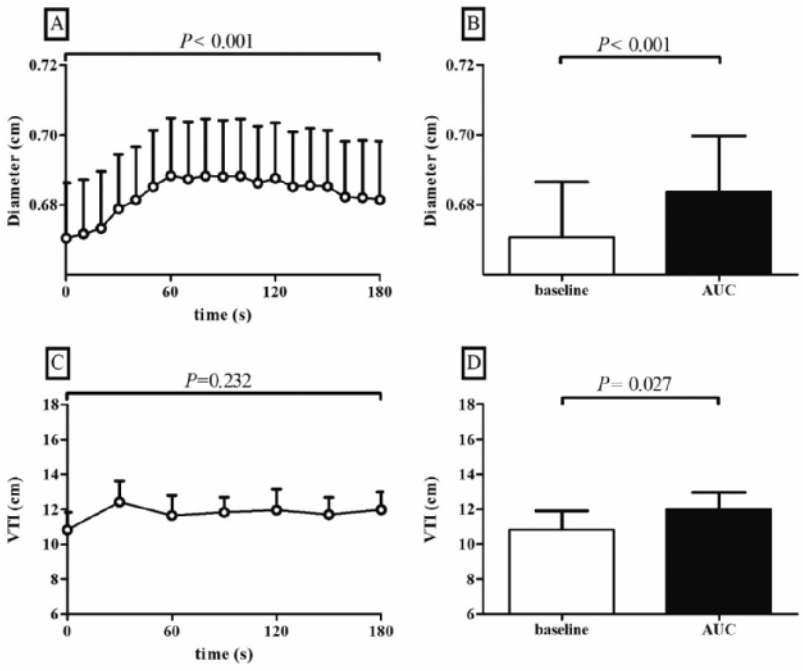

Figure 1. Responses of the carotid artery and the LAD coronary artery to CPT.
A. Diameter change of the carotid artery over time. B. Percentage change in carotid diameter at baseline and during CPT (area under the curve, AUC). C. VTI change of the LAD coronary artery over time. D. Percentage change in LAD coronary artery VTI at baseline and during CPT (area under the curve, AUC). Data are presented as mean, error bars represent standard error of the mean (SEM). White bars represent baseline measurements, black bars represent peak values.

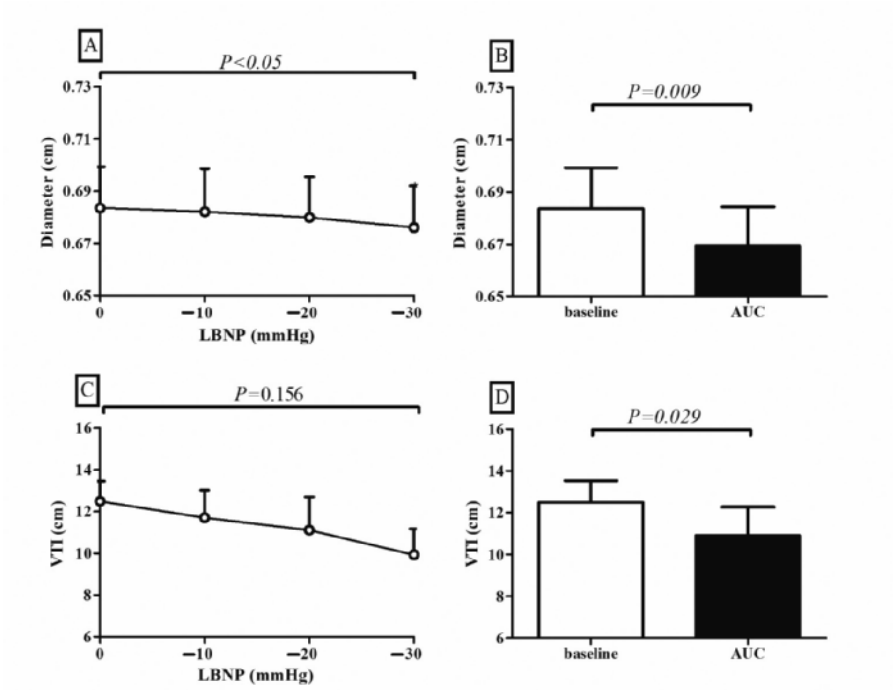

Figure 2. Responses of the carotid artery and the LAD coronary artery to LBNP.
A. The diameter change of the carotid artery over time. B. The percentage change in carotid diameter at baseline and during LBNP. C. the VTI change of the LAD coronary artery over time. D. The percentage change in LAD coronary artery VTI at baseline and during LBNP. Data are presented as mean, error bars represent standard error of the mean (SEM). White bars represent baseline measurements, black bars represent peak values.

Carotid artery response to sympathetic activation: role of α_1-receptors.

Cold pressor test. Prazosin increased baseline CCA diameter, and decreased shear (0.671±0.05 to 0.703±0.05 cm, and 185.9±50 to 159.1±40 s^{-1} respectively, all P<0.05), whilst no changes were found in carotid blood flow and blood velocity (10.9±1.8 to 10.8±1.5 ml/sec P=0.405, and 30.7±6.6 to 27.7±5.2 cm/sec, P=0.051). Prazosin caused an abolished CPT-induced increase in diameter (Figure 3). Prazosin attenuated the increase in blood pressure during CPT, and resulted in a larger increase in cardiac output, heart rate and RPP during the CPT (n=9, Table 2). We found no change in stroke volume (Table 2), whilst we also found no change in CCA flow, shear and velocity (data not shown).

Lower-body negative pressure. Baseline CCA diameter, flow and velocity were significantly larger following Prazosin administration (0.684±0.05 to 0.706±0.05 cm, 10.2±1.6 to 12.0±2.3 ml/sec, and 27.6±5.0 to 30.0±5.5 cm/sec, respectively, all P<0.05). All subjects reached pre-

syncope at -30 or -40 mmHg. Therefore, we compared data between both sessions up to -30 mmHg. Prazosin did not alter CCA diameter responses during LBNP (Table 2, Figure 4). Prazosin exaggerated the increase in heart rate and RPP during LBNP, whilst blood pressure decreased during the Prazosin trial (n=9, Table 2).

Carotid artery responses versus coronary artery responses.

SNS stimulation. Similar to CCA responses, CPT caused a significant increase in LAD maximum velocity (n=6, baseline 0.25±0.03 to peak 0.34±0.02 cm/sec, *P*<0.05) and VTI (*P*<0.05, Figure 1). Due to the suction of the LBNP box, movement of the participants prevented assessment in 5 participants. Again in agreement with CCA responses, LBNP caused a reduction in LAD maximum velocity (n=5, Table 2) and peak VTI (cm, Figure 2). When pooled, a significant correlation was found between changes in CCA diameter and LAD peak VTI (n=20, r=0.65, *P*<0.01).

α_1-*receptor blockade.* Following Prazosin administration, LAD VTI were elevated and Prazosin abolished the increases in CCA diameter and LAD peak VTI (Figure 3, Table 2). During LBNP, Prazosin did not alter CCA diameter (cm), LAD peak VTI or LAD peak velocity responses (cm/sec, up to -30 mmHg; Table 2, Figure 4).

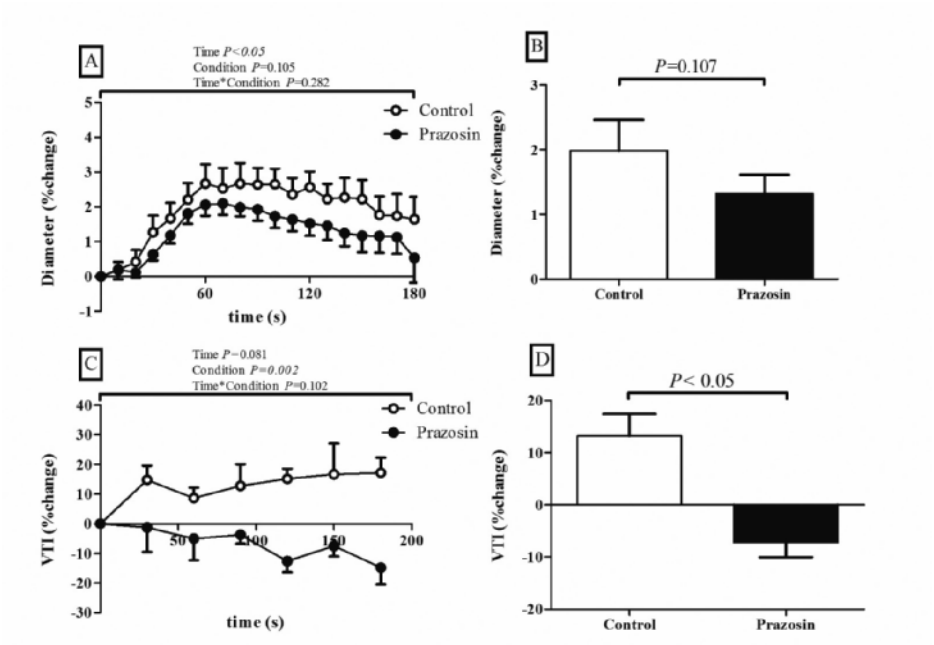

Figure 3. Responses of the CCA and the LAD to CPT, Control versus Prazosin condition.
A. Diameter change of the carotid artery over time. B. Percentage change in diameter. C. VTI change of the LAD coronary artery over time. D. Percentage change in LAD coronary artery VTI at baseline and during the CPT. Data are presented as mean, error bars represent standard error of the mean (SEM). White bars represent the *Control* condition, black bars represent the *Prazosin* condition.

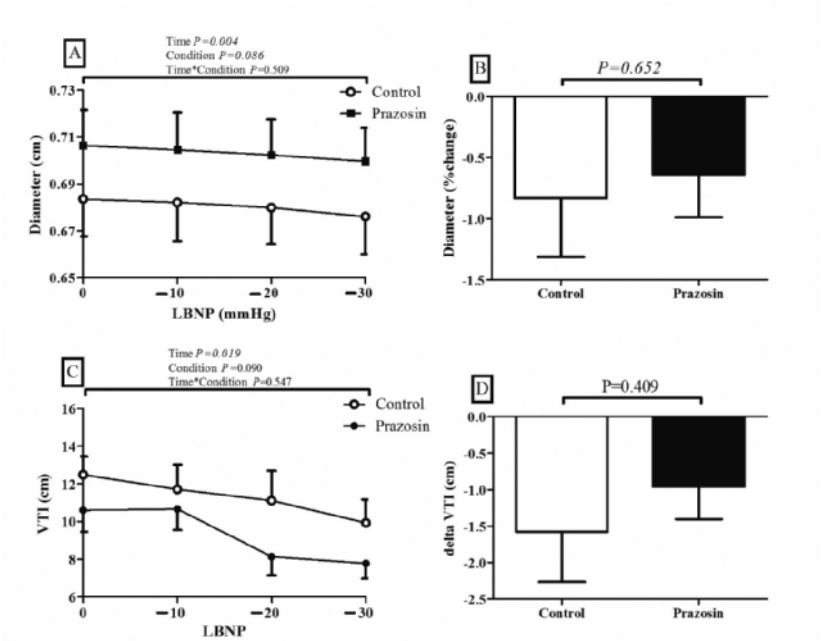

Figure 4. Responses of the CCA and the LAD to LBNP, Control versus Prazosin condition.
A. Diameter change of the carotid artery over time. B. Percentage change in carotid diameter at baseline and during the LBNP. C. VTI change of the LAD coronary artery over time. D. Percentage change in LAD coronary artery VTI at baseline and during the LBNP. Data are presented as mean, error bars represent standard error of the mean (SEM). White bars represent the *Control* condition, black bars represent the *Prazosin* condition.

Discussion

We present the following findings. First, activation of the SNS using the CPT significantly increased CCA diameter, whilst SNS activation using LBNP mediated a decrease in CCA diameter. Second, systemic blockade of the α_1-receptors significantly attenuated the dilator response of the carotid during the CPT, whilst these changes were unaltered during LBNP. This latter finding suggests the presence of distinct carotid artery responses to different types of SNS activation, with a distinct contribution of α_1-receptors mediating these responses. Furthermore, we found good agreement between the direction and magnitude of the coronary and carotid artery responses when comparing the different tests of sympathetic stimulation, but also regarding the contribution of α_1-receptors. Taken together, we found divergent responses to distinct tests of SNS activation and the role of α_1-receptors mediating these responses, whilst similarity is found between carotid and coronary arteries in the magnitude and direction of vascular responses to sympathetic stimulation and blockade of α_1-receptors.

Carotid artery responses to sympathetic stimulation.

The CPT resulted in a characteristic dilation in the CCA of our healthy subjects, a finding observed previously in our laboratory[12] and others.[11] Interestingly, these dilator responses of the carotid artery contrasts with peripheral artery responses, since brachial or superficial femoral arteries demonstrate negligible diameter changes during CPT.[14, 16] Central, elastic arteries (such as the carotid artery) may thus respond differently to SNS activation using the CPT compared to muscular, peripheral arteries. This notion is further supported by observations of abdominal aorta dilation during the CPT.[38] In contrast, LBNP mediated a decrease in CCA diameter. The presence of distinct artery responses to different tests of sympathetic activation has also been reported in peripheral conduit arteries.[14] Both CPT and LBNP mediate sympathetic activation through different pathways, leading to distinct vascular responses in peripheral and central arteries. The CPT causes an immediate stressor response,[5, 14] leading to rapid catecholamine release and blood pressure elevation. This induces β-receptor mediated vasodilation, and sympathetic blood pressure mediated constriction, respectively. The resultant of this response is an increase in CCA diameter, due to the outweighing effect of β-receptor mediated vasodilation. In contrast, the LBNP mediates a gradual, arterial baroreflex-mediated activation of the sympathetic nervous system and thus can directly decrease carotid diameter. Both sympathetic tests demonstrate distinct time-dependent changes in circulating catecholamines, with an immediate elevation after CPT, and a slower (time- and intensity-dependent) elevation during LBNP.[5, 14, 24, 39] This data indicates that distinct tests of stimulation of the sympathetic nervous system lead to different carotid artery responses.

Role of α_1-receptors in carotid artery responses to sympathetic stimulation.

Under physiological conditions, α_1-receptors mediate vasoconstriction in coronary arteries during a sympathetic stimulus.[20, 40] Indeed, blockade of α_1-receptors resulted in an increase in baseline CCA diameter and velocity, but also LAD velocity. However, in contrast to our hypothesis, α_1-blockade attenuated the carotid artery dilator responses during the CPT, whilst no impact of α_1-blockade was found during LBNP. One potential explanation is that the increase in baseline diameter and/or velocity (induced by α_1-receptor blockade) prevented a further increase in diameter upon additional SNS stimulation. This explanation is supported by previous work in peripheral arteries, which found that an increase in baseline diameter is associated with a smaller endothelium-(in)dependent vasodilation.[41, 42] However, our data does not reveal such a relation between resting carotid diameter and peak responses (CPT control r=-0.280, Prazosin r=-0.275, LBNP control r=-0.401, Prazosin r=-0.219, all P>0.05). Therefore, CCA dilation during α_1-blockade may not explain the attenuated vasomotor responses to CPT or preserved response to LBNP.

An alternative explanation for the attenuated dilator response may relate to the pharmacological actions of α_1-receptor blockers. In healthy coronary arteries, vasoconstriction upon sympathetic stimulation is largely mediated via α_1-receptors, with only a minor role for α_2-receptors.[43, 44] Previous studies in both animals and humans found that during α_1-receptor blockade, SNS activation still mediates coronary constriction through activation of α_2-receptors.[45-47] Possibly, α_1-receptor blockade in our study yielded stimulation of α_2-receptors *during* activation of the SNS using the CPT. Consequently, the vasodilator responses may be attenuated by the constrictive actions of α_2-receptors. This hypothesis needs further exploration. A final reason for the diminished CCA dilation during CPT could reside in the attenuated blood pressure responses. However, it is unclear whether blood pressure represents the principal contributor to the carotid dilation, especially since peak diameter responses precede peak blood pressure values. Moreover, blood pressure rises similarity between individuals who demonstrate carotid artery vasodilation *versus* vasoconstriction.[12] Nonetheless, we cannot exclude a potential role for the blood pressure response to contribute to the carotid dilation.

Carotid artery *versus* coronary artery.

Our findings provide strong evidence for similarity between the carotid and coronary arteries regarding the direction and magnitude of the vasomotor response. Indeed, both carotid and coronary arteries demonstrated dilation in response to CPT, but constriction was present in both arteries during LBNP. The presence of coronary dilation to CPT,[6, 7] but also coronary constriction to LBNP[5] has been reported in previous studies. This further confirms the presence of distinct artery responses to distinct stimuli to activate the sympathetic nervous system. Furthermore, α_1-receptor blockade mediated similar effects between carotid and coronary arteries for the LBNP test. During the CPT we observed that α_1-receptor blockade attenuated the carotid responses, whilst the coronary responses were reversed. Agreement between arteries was further supported by the presence of a significant and strong correlation between both arteries (Figure 5), a finding that is in line with previous work.[11, 12] A potential limitation of the echocardiographic measurement is the inability to examine blood flow. However, strong agreement is present between changes in coronary artery blood velocity and blood flow in response to sympathetic stimulation,[4, 5, 48] suggesting that the increase in LAD velocity can be interpreted as true coronary vasodilation.

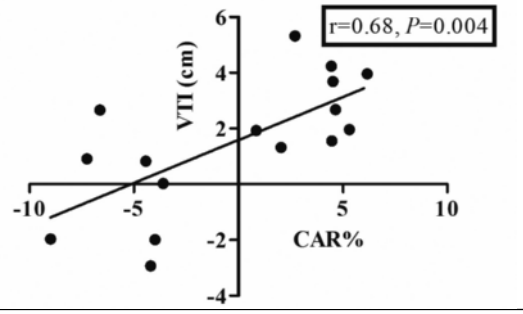

Figure 5. Correlation between the carotid artery diameter and coronary LAD response
Correlation between the carotid artery diameter response (CAR%) and coronary LAD response (change in the velocity time integral (VTI in cm)) pooled for the cold pressor test and lower body negative pressure test (n=16).

Despite these similarities in magnitude and direction of vascular responsiveness, it is important to emphasise that the mechanisms contributing to vascular control may differ between arteries. For example, coronary artery flow and velocity during sympathetic stimulation are dependent on both local metabolic and vasodilatory mechanisms sensitive to the rate of myocardial oxygen consumption (MVO_2).[5] For this purpose, we have calculated the rate-pressure product, a common used index for myocardial oxygen consumption (RPP, Figure 6). The increase in RPP during the CPT suggests that the dilation of the coronary artery is, at least partly, related to the increase in myocardial oxygen uptake. Whether similar mechanisms are present in the brain to contribute to carotid artery dilation during the CPT is currently unknown. For the LBNP, we found no important role for RPP to contribute to the vascular responses in our study. When correcting our responses for potential differences for the RPP, correlation between LAD VTI and CAR(%) remained present (r=0.66, P<0.05). Future studies are required to better understand the mechanisms contributing to the vascular responses during sympathetic stimulation in both carotid and coronary arteries.

Clinical relevance. Coronary artery responsiveness to SNS stimulation, including the CPT, has shown a strong predictive ability for future cardiovascular disease and/or events.[2, 9, 10] Similarity in vasomotor responsiveness between coronary and carotid arteries suggests that the carotid artery may serve as a alternative measure for coronary vascular responses to SNS stimulation. An important advantage of measuring the carotid artery is its easy accessibility, high reproducibility and the accuracy of the test. This warrants future studies to further explore the potential clinical use of examining carotid responses to SNS stimulation. To further explore the similarity between the carotid and coronaries, future studies could be performed in a catheterisation laboratory, to simultaneously measure both carotid and coronary artery responses during sympathetic stimulation. These studies can be extended by the addition

of selective α- and /or β-adrenergic agonist/antagonists, to further resolve the contribution of adrenergic receptors to sympathetically-mediated carotid and coronary artery responses.

Methodological considerations. A strength of our study was that we controlled for end-tidal gases at baseline values, during both CPT and LBNP, and the α_1-receptor blockade condition. Fluctations and alterations in $P_{ET}CO_2$ are known to directly influence the diameter of the CCA[49] and LAD VTI.[29] Following our α1 blockade, which directly affects mean arterial pressure and ventilatory regulation during sympathetic activation, clamping $P_{ET}CO_2$ and $P_{ET}O_2$ to baseline values reduced the possible interference with our carotid and coronary artery responses.

To summarize, our data demonstrates that the carotid artery demonstrates distinct vascular responses to different stimuli to activate the sympathetic nerve system. Additionally, blockade of the α_1-receptors significantly attenuated the dilator responses in the carotid artery during the CPT, whilst no changes were found during LBNP, suggesting a potential role for α-receptors to contribute to vasomotor responses in carotid arteries. Finally, even though α_1-blockade resulted in disparate responses during CPT, our findings indicate strong similarity between carotid and coronary artery reactivity in response to distinct sympathetic stimuli.

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Table 1. Cold pressor test, hemodynamic responses

Cold pressor test		1 minute CPT						2 minutes CPT		
	Baseline	**10**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**
Stroke volume (ml) Control	105±15	106±17	106±16	105±17	105±17	105±19	105±20	105±21	104±20	104±20
Stroke volume (ml) Prazosin	112±14	113±15	114±14	114±15	113±16	112±17	111±16	110±15	111±17	111±16
Cardiac Output (L/min) Control	6.2±1.3	6.6±1.2	6.8±1.3	6.6±1.4	6.7±1.6	6.8±1.8	7.0±1.6	6.9±1.6	6.7±1.7	6.6±1.5
Cardiac Output (L/min) Prazosin	6.7±0.9	7.4±1.0	7.5±1.2	7.5±1.3	7.7±1.5	7.7±1.6	7.5±1.6	7.6±1.6	7.5±1.4	7.5±1.4
Heart Rate (bpm) Control	59±12	63±10	65±11	64±11	64±13	64±12	67±12	66±12	64±13	63±13
Heart Rate (bpm) Prazosin	62±12	69±13	68±11	68±11	71±13	71±14	70±13	71±14	70±15	70±14
Diastolic BP (mmHg) Control	79±8	83±6	81±7	84±9	86±8	88±8*	91±8*	92±9*	92±8	92±8
Diastolic BP (mmHg) Prazosin	74±9	76±9	74±9	76±9	78±9	80±9	81±10	81±9	81±9	82±8*
Systolic BP (mmHg) Control	133±7	139±8	137±10	140±11	142±12	146±13	148±11*	150±12*	150±12*	150±13
Systolic BP (mmHg) Prazosin	131±7	136±6*	135±7	136±7	138±6	140±7	141±9	140±7	140±7	140±6
Rate pressure product - Control	7927±1754	8787±1449	8963±1654	9028±1804	9160±2048	9483±2096	9901±2047	9978±2093	9619±2114	9549±2097
Rate pressure product - Prazosin	8141±1534	9380±1761	9169±1724	9284±1750	9771±1778	9953±2062	9833±2023	9960±2072	9887±2196	9858±2024
LAD velocity max Control	0.252±0.03			0.325±0.07			0.304±0.03			0.280±0.05
LAD velocity max Prazosin	0.261±0.04			0.312±0.04			0.302±0.07			0.281±0.06
LAD velocity mean Control	0.201±0.03			0.256±0.07			0.232±0.03			0.224±0.04
LAD velocity mean Prazosin	0.199±0.03			0.25±0.02			0.226±0.03			0.228±0.05

					3 minutes CPT				2-way ANOVA		
100	**110**	**120**	**130**	**140**	**150**	**160**	**170**	**180**	**Time**	**Trial**	**Time* Trial**
103±20	105±21	106±21	106±21	106±20	106±20	106±20	107±19	107±19	0.450	0.296	0.450
109±17	110±17	110±17	111±17	112±17	111±17	111±18	112±18	112±17			
6.3±1.4	6.3±1.5	6.3±1.4	6.3±1.5	6.1±1.6	6.3±1.5	6.3±1.4	6.3±1.5	6.3±1.4	0.200	*0.049*	*0.021*
7.5±1.4	7.6±1.3	7.6±1.2	7.4±1.1	7.6±1.1	7.6±1.1	7.6±1.0	7.7±0.9	7.6±1.0			
62±12	62±13	61±12	61±13	59±15	61±13	61±12	60±14	59±12	0.107	*0.024*	*0.001*
71±14	72±14	72±14*	70±13	71±13*	71±12	71±13*	71±12*	70±13*			
93±9*	92±9*	92±9*	92±9*	91±8*	90±8*	90±8*	90±8	89±8*	*0.000*	*0.045*	*0.031*
82±9*	82±8	83±9	80±9	81±9*	82±9*	81±9*	81±9*	80±8*			
151±13	150±13	151±12	151±11*	150±12*	149±11*	148±10*	148±11	148±11*	*0.000*	0.169	*0.023*
141±8	141±8	141±9	139±9	140±8	141±9*	141±9*	140±9*	140±7*			
9458±1931	9253±2024	9212±1982	9166±2045*	8890±2087	9077±2011	8984±1819	8883±2042	8834±1927	*0.008*	0.307	*0.014*
10088±2018	10158±2138	10217±2204	9705±1855	9892±1870*	9941±1667*	9981±1580*	9928±1482*	9826±1709*			
		0.261±0.04			0.266±0.05			0.265±0.05	0.007	0.769	0.594
		0.278±0.06			0.284±0.06			0.279±0.05			
		0.209±0.03			0.215±0.03			0.207±0.03	0.041	0.603	0.400
		0.231±0.04			0.231±0.04			0.228±0.04			

Hemodynamic and coronary responses during Cold pressor test (averaged per 10 second intervals). P-values refer to 2-way repeated measures ANOVA's, for within participant comparison (time), between trial comparison, and the interaction time*trial. *Symbols denote P<0.05 difference to baseline values.

Table 2. Lower body negative pressure, hemodynamic responses

Lower body negative pressure								2-way ANOVA		
	Baseline	-10		-20		-30		Time	Trial	Time*Trial
Stroke volume (ml) Control	106±13	102±12	100±12	97±12	97±13	93±14	90±14	*0.000*	0.687	0.086
Stroke volume (ml) Prazosin	106±12	103±14	99±16	96±15	93±16	91±16	85±18*			
Cardiac Output (L/min) Control	6.4±1.2	6.1±1.1	6.1±1.1	5.9±1.1	6.0±1.0	5.8±1.0	5.9±1.0	*0.642*	*0.002*	0.162
Cardiac Output (L/min) Prazosin	7.1±1.4	7.1±1.2	7.2±1.4	7.2±1.0	7.4±0.9	7.5±0.8	7.3±0.9			
Heart Rate (bpm) Control	61±12	60±12	61±13	62±13	63±13	64±14	67±14	*0.000*	*0.002*	*0.009*
Heart Rate (bpm) Prazosin	68±16	70±16	75±20	77±17*	82±17*	84±17*	89±17*			
Diastolic BP (mmHg) Control	77±9	77±9	77±9	78±9	78±9	79±9	79±10	*0.177*	0.160	0.060
Diastolic BP (mmHg) Prazosin	73±8	74±8	72±9	74±8	73±8	74±9	73±9			
Systolic BP (mmHg) Control	132±7	131±6	129±7	130±7	131±6	130±7	131±7	0.152	0.388	*0.005*
Systolic BP (mmHg) Prazosin	131±9	131±9	128±9	129±9	127±10	127±10	124±11			
Rate pressure product - Control	8048±1746	7923±1745	7929±1831	8024±1766	8192±1741	8348±1895	8708±1916	*<0.001*	*0.027*	*0.025*
Rate pressure product - Prazosin	8954±2618	9253±2537	9646±2963	9969±2491*	10479±2585*	10751±2568*	10960±2319*			
LAD VTI Control	12.1±2.7	11.1±3.6		9.9±3.4		8.7±1.3		*0.019*	0.09	0.547
LAD VTI Prazosin	9.5±0.9	9.8±1.9		8.1±2.2		7.8±1.8				
LAD velocity max Control	0.277±0.05	0.26±0.06		0.243±0.05		0.226±0.03		*0.029*	0.52	0.621
LAD velocity max Prazosin	0.25±0.06	0.249±0.03		0.226±0.04		0.225±0.04				
LAD velocity mean Control	0.21±0.03	0.19±0.03		0.203±0.04		0.187±0.03		0.496	0.973	0.177
LAD velocity mean Prazosin	0.197±0.03	0.207±0.02		0.188±0.03		0.20±0.03				

Hemodynamic and coronary responses during Lower body negative pressure test (averaged per 2 minute stages). P-values refer to 2-way repeated measures ANOVA's, for within participant comparison (time), between trial comparison, and the interaction time*trial. *Symbols denote P<0.05 difference to baseline values.

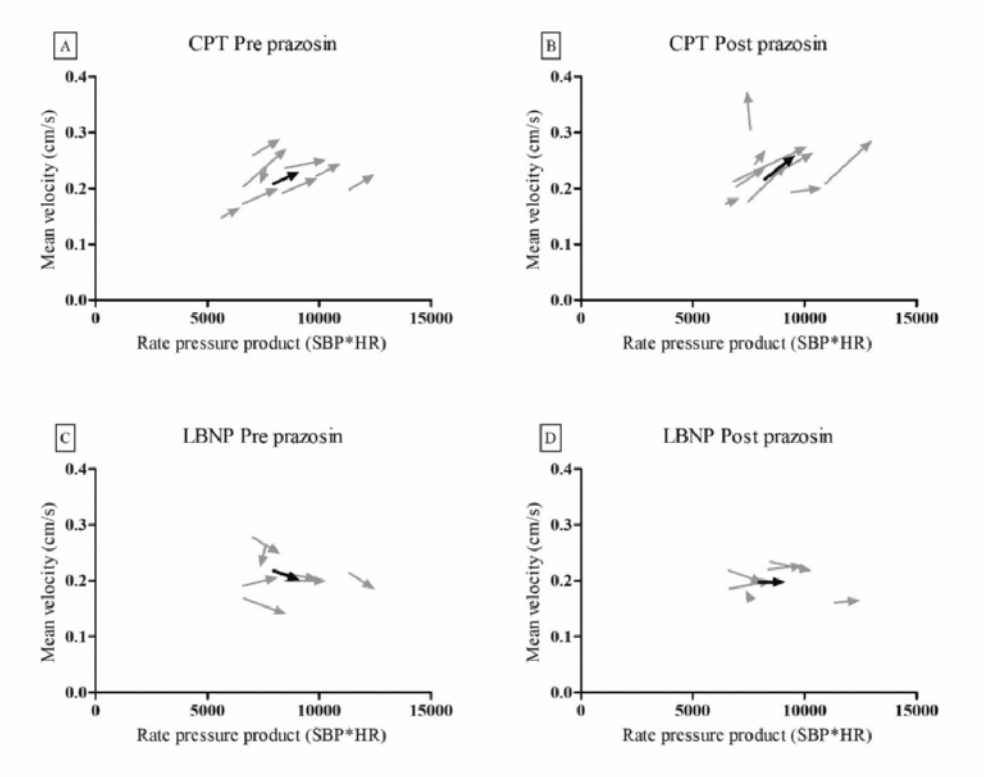

Figure 6. Mean coronary velocity expressed versus the rate pressure product (RPP).
ΔA. Responses of mean coronary velocity versus RPP during CPT. B. Responses of mean coronary velocity versus RPP during CPT with prazosin. C. Responses of mean coronary velocity versus RPP during LBNP. D. Responses of mean coronary velocity versus RPP during LBNP with prazosin. Light grey indicates individual responses, whilst the black arrows indicate the mean response.

Chapter 6

Carotid artery reactivity predicts events in peripheral arterial disease patients.

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Abstract

Objective. Patients with peripheral arterial disease (PAD) have increased risk on future cerebro- and cardiovascular (CV) events. Our aim was to examine whether the carotid artery reactivity (CAR; a novel, simple procedure to examine endothelial function) predicts CV events in PAD patients.

Methods. 172 PAD patients (68±10 years, 67% male) underwent the CAR, which involves ultrasound measurement of carotid artery diameter during sympathetic stimulation produced by 90sec hand immersion in 4°C ice-water (i.e. cold pressor test). CAR-responses were dichotomised into carotid constriction or dilation. We recorded cardiac and cerebrovascular events, mortality and clinical progression to percutaneous transluminal angioplasty or loss of patency during 12-months follow-up.

Results. Eighty-two PAD patients demonstrated carotid constriction and 90 patients demonstrated dilation. PAD patients with carotid constriction showed more (CV) events compared to patients with dilation (Kaplan-Meier Log rank; $P<0.05$). Cox proportional hazard model showed that patients with carotid constriction continued to show higher risk for CV events (Hazard Ratio: 4.1, 95%CI: 1.3-12.5) and clinical progression (Hazard Ratio: 2.0, 95%CI: 1.2-3.3), even after adjustment for other risk factors. Ankle brachial pressure index and carotid intima-medial thickness alone did not predict (CV) event or improve risk assessment beyond that provided by CAR.

Conclusion. Carotid vasoconstriction identifies PAD patients with a four-fold increased risk for future CV events and two-fold increased risk for clinical deterioration. CAR provides a simple, novel strategy to predict CV events and progression in PAD patients.

Introduction

Peripheral arterial disease (PAD) is the result of atherosclerotic arterial stenosis and occlusions in the larger vessels supplying the lower extremities.[1] Patients with PAD have a markedly increased risk for future cardiovascular mortality and morbidity.[1-4] Endothelial dysfunction contributes to the development and progression of PAD.[5, 6] Endothelial function, often examined as the brachial artery reactivity response to ischaemia, in PAD patients is impaired[7-9] and relates to future CV events.[10, 11] The recent AHA/ACC-guideline on the management of PAD highlighted the need for an easy, simple and rapid test of endothelial function to predict future adverse events in PAD.[12] Although brachial artery reactivity shows predictive capacity for future CV events,[10, 11] concerns have been raised regarding practical limitations that prevents the clinical application of this technique.

Carotid artery reactivity (CAR) testing is a simple, non-invasive procedure to examine endothelial function. It involves measuring the carotid artery diameter responses to the sympathetic stimulation produced by the cold pressor test (CPT).[13, 14] The carotid arteries, like coronary arteries,[13, 14] dilate in response to the CPT in healthy subjects, whereas this dilation is attenuated, or reversed to vasoconstriction, in patients with cardiovascular disease.[13-16] Interestingly, the coronary arteries' response to CPT is a strong, independent predictor of cardiovascular events,[17, 18] but it is not clear if the carotid response to CPT (i.e. CAR) also predicts future cardiovascular events. This study is to our knowledge, the first to examine the prognostic value of the CAR in patients with PAD. We hypothesized that CAR-induced vasoconstriction would predict future CV events in patients with PAD, independent of subject characteristics and clinical status.

Methods

Participants and study approval

We recruited 172 patients with PAD scheduled for a routine visit at the vascular laboratory (Department of Surgery, Radboud University Medical Center, Netherlands) for the study (Figure 1). We included PAD patients with present or prior Fontaine classification 2B-3-4, age ≥18yr, and the ability to provide informed consent. We excluded patients with Raynaud's phenomenon, chronic pain syndrome, open wounds on the upper extremities, arterial-venous shunts, scleroderma, coronary, central and/or peripheral arterial disease interventions within the prior <1 week, and unstable angina pectoris, myocardial infarction, stroke or heart failure within the prior 3 months. Patients provided written informed consent prior to participation. The study was approved by the local Ethics Committee (NL-46109.091.13) in accordance with the latest revision of the Declaration of Helsinki. This study was registered as NTR-4117 (Netherlands Trial Registration).

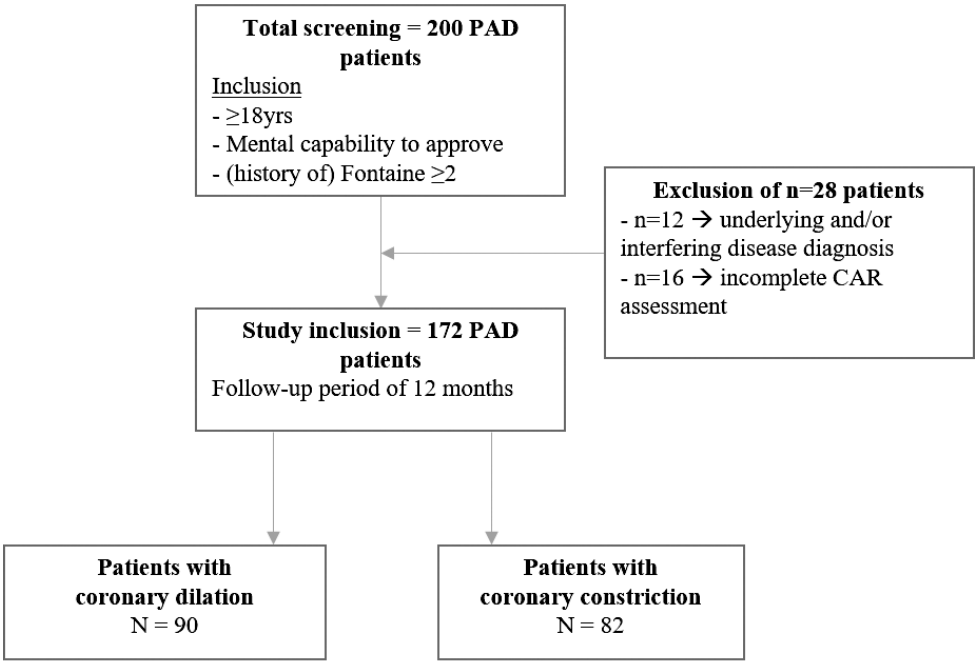

Figure 1. Consort diagram

Experimental design

Patients abstained from strenuous exercise for 24 hours, fasted for ≥6 hours, and abstained from caffeine and vitamin C, which are known to alter endothelial function, for ≥18 hours prior to testing in accordance with guidelines on assessing endothelial function.[20]

Experimental measures

General characteristics. Age and sex were obtained from the electronic patient records. A physician obtained the history of smoking, hypercholesterolemia, hypertension, diabetes and medication use. Height (in m), weight (in kg), and waist-hip ratio (WHR) were measured by a research nurse. The same vascular surgeon assigned the patient's Fontaine clinical classification. Patients with Fontaine stage ≥2 performed walking tests to distinguish between Fontaine stages. Finally, the ankle-brachial pressure index (ABPI) was measured based on clinical requirements following recent guidelines.[21] The highest systolic pressure in the right and left posterior tibial or dorsal pedis artery and in the right brachial artery was measured twice, and the average of those two measurements was used to calculate the ABPI for

each leg. The lowest ABPI of two legs was used for analysis. For the purpose of analysis, we compared those above versus below the median ABPI.

Carotid artery reactivity. Patients rested on a comfortable bed in a temperature controlled room for at least 5 minutes. Participants were in the supine position with the neck extended for assessment of the carotid artery. Left carotid artery diameter was recorded continuously for 30-seconds before and for 90-seconds during immersion of the hand up to the wrist in ice slush (4 °C). Images were obtained using a L9-3 MHz linear array probe attached to a high resolution ultrasound machine. When an optimal image was found, the probe was held stable and the ultrasound parameters were set to optimise the longitudinal, B-mode image of the lumen-arterial wall interface. Following a 30-second baseline assessment of carotid artery diameter, the hand was immersed for 90-seconds with simultaneous and continuous assessment of carotid artery diameter.

CAR% responses were assessed for diameter. Analysis of the carotid artery diameter was performed by a single blinded investigator using custom-designed edge-detection and wall-tracking software, which is largely independent of investigator bias.[22] Details of this technique can be found elsewhere.[23] Baseline diameter was calculated as the mean of data acquired across the 30 seconds preceding the CPT test. After submersion of the hand in ice slush, data were calculated as the mean value for 10-second intervals, involving 8-10 full cardiac cycles. Based on this data we calculated the peak diameter change (i.e. the 10-second bin with the highest value, CAR%). The peak diameter change can refer to a maximum constriction or dilation. The direction of this change was determined by a positive (i.e. dilation) or negative (i.e. constriction) area under the curve.

Reproducibility (coefficient of variation, CV) of diameter responses to CPT were previously assessed with a 1- and 24-hour intervals in 50 subjects. Within-day CV for baseline and peak diameters were 2.2 and 2.6%, whilst day-to-day CV were 2.3% and 2.7%. Furthermore, the CAR% (i.e. maximum change in diameter) showed a within-day reproducibility of 2.6% and between-day reproducibility of 2.8%.[14]

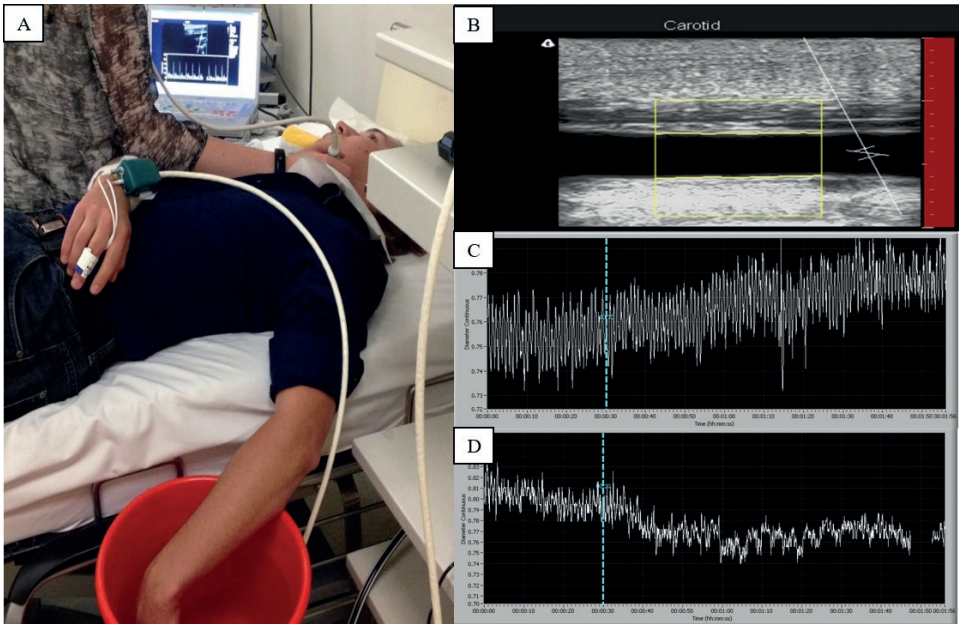

Figure 2. Study set-up

Set-up of the practical performance of the test (A). Screen-shot of the assessment of carotid artery diameter, with the yellow box indicating the region of interest within the automated software performed analysis of the diameter (i.e. yellow lines on the artery wall) (B). Data from a representative subjects demonstrating carotid artery diameter dilatation (C) and constriction (D) during the cold pressor test. Both panels represent the carotid artery diameter (in cm) across the 30-s baseline (up to the vertical dashed line; the start of the cold pressor test) and 90-s during the cold pressor test. Data were analysed in 10-second bins to identify presence of dilatation or constriction. More detailed findings of this procedure is presented in the methods section.

Intima-media thickness. Carotid artery intima-media thickness (cIMT), a marker for vascular structure, is related to future development of PAD.[24] To examine whether the CAR relates to future CV events, independent of the cIMT, we examined cIMT from the same section of the artery as the CAR. We obtained continuous recordings of the cIMT for 10 seconds. Analyses were performed by a blinded researcher, using observer-independent edge-detection and wall-tracking software.[25] For the purpose of analysis, we compared those above versus below the median cIMT.

Follow up and assessment of adverse events

After 12 months of follow-up, adverse events were extracted from medical records and verified by a blinded vascular surgeon (MW). The Dutch National Death Registration was used to determine mortality. Death certificates of patients who experienced a fatal event were obtained when available to categorise death into CV or non-CV related mortality. Adverse events were categorised into; 1. Cardio- and cerebrovascular events ("CV events"; CV-related mortality, myocardial infarction, coronary revascularisation procedures, transcranial ischemic attack, cerebrovascular accident, carotid surgery, major- and minor amputations, and ischemic bowel disease), 2. "Clinical progression" that is related to PAD (loss of patency (i.e., the presence of restenosis in a previous endovascular reconstructed vessel), endovascular reconstructive surgery using percutaneous transluminal angioplasty, and worsening in Fontaine-classification), and 3. "All-cause Mortality". We also grouped these 3 categories to capture all adverse events ("Adverse events"). Only the first event was included in the analyses for patients who experienced more than 1 event. All indications for PTA and revascularisation surgery were discussed prior in a multidisciplinary team of vascular surgeons and interventional radiologists, whilst preference of patient and interventional were taken into consideration. All involved members of the multidisciplinary team were blinded to the outcome of the CAR test.

Statistical analysis

Prior to our study, we aimed to include 200 PAD patients. This group size is in line with previous studies examining the prognostic value of measures of vascular health,[10, 19] whilst this group size also accounts for potential drop out (10-15%) and access to sufficient PAD patients (n=400/annum, 50% inclusion rate). Data are presented as mean±SD or n (%) unless stated otherwise. Statistical analysis was performed using IBM SPSS Statistics 21.0 (IBM SPSS, IBM Corp., Armonk, NY, USA). Baseline characteristics were assessed for normality with the Shapiro-Wilk test. We adopted unpaired Student's t-tests (Mann-Whitney U-test for non-normally distributed parameters) to compare subject characteristics, co-morbidities, clinical status, and medication use between PAD patients with carotid constriction vs. dilation. We used logistic regression to assess if subject characteristics, co-morbidities, clinical status, or medication could predict presence of carotid constriction.

Analyses to examine whether presence of carotid constriction could predict future events, analyses were performed separately for "CV events", "Clinical progression", "All-cause mortality" and "Adverse events". Cumulative event rates of carotid constriction and dilation were estimated with Kaplan-Meier survival analyses, and were calculated with the log-rank test. Cox proportional hazard models were used to calculate hazard ratios, including correction for confounding variables (age, sex, BMI, and WHR). These variables were selected based on prior evidence of an association with measures of endothelial function.[14, 20] Analyses were repeated in subgroups in whom we examined cIMT (n=169) or ABPI (n=142). We examined if

these clinical measures were related to increased risk for future events,[26] and whether they altered the analyses related to carotid measurement.

Results

Subject characteristics, co-morbidities, clinical status and adequate imaging was obtained in all 172 PAD patients. Median change in carotid artery diameter was 0.8% (95% Confidence Interval -19.3 – 11.6%). Carotid constriction occurred in n=82 patients, whereas vasodilation occurred in 90 patients. Seventy patients (41%) experienced ≥1 event, which included loss of patency (n=18), increase in Fontaine-classification (n=15), percutaneous transluminal angioplasty (n=44), transient ischemic attack (n=1), myocardial infarction (n=4), cerebrovascular accident (n=3), coronary revascularisation procedures (n=1), major (n=7) and minor (n=1) amputation, and ischemic bowel disease (n=1). Ten PAD patients died; from CV-related mortality (n=4), cancer-related mortality (n=2), and unknown cause (n=4). We found no baseline differences between PAD patients with carotid constriction versus dilation in subject characteristics or co-morbidities. Patients with carotid constriction reported lower antiplatelet drugs usage (*Table 1*). Logistic regression revealed that none of the subject characteristics, co-morbidities, clinical status or medication use could predict if a PAD patient would demonstrate carotid constriction (backward likelihood ratio analysis, all parameters P>0.05). When comparing patients with and without an adverse event, those with events showed higher prevalence of hypercholesterolemia. Other factors did not differ between groups (*Supplemental data 1*).

Prognostic value of carotid constriction for future adverse events.

Kaplan-Meier survival curves demonstrated that PAD patients with carotid constriction report a higher incidence of CV events (*P*=0.007), clinical progression (*P*=0.005) and adverse events (*P*=0.006) compared to carotid dilation (*Figure 3*). There were no significant differences between groups for all-cause mortality (Log-rank, *P*=0.417, *Figure 3*).

Using a multivariate Cox proportional hazard model, with the fully adjusted model correcting for potential confounders (i.e. age, sex, WHR and BMI), PAD patients with carotid constriction continued to demonstrate higher risk for CV events (HR 4.1, 95%CI 1.3-12.5), clinical progression (HR 2.0, 95%CI 1.2-3.3) and adverse events (HR 1.8, 95%CI 1.1-3.0), but not all-cause mortality (HR 1.4, 95%CI 0.4-5.1, *Table 2*).

Table 1. Baseline characteristics

	Total group	CAR constriction	CAR dilation	P-value
Subject characteristics	n=172	n=82	n=90	
Age*, y	68±10	71 [43-85]	67 [46-90]	0.223
Sex, males (%)	115 (67)	58 (71)	57 (63)	0.303
Height (m)	1.73±0.09	1.73±0.10	1.72±0.10	0.464
Weight (kg)	79.8±14.6	80.1±14.3	79.6±14.9	0.804
Body-mass index (kg/m2)	27±4	27±4	27±4	0.900
Waist-to-hip ratio	1.01±0.10	1.02±0.10	1.00±0.10	0.210
Smoking, yes n (%)	55 (32)	28 (34)	27 (30)	
History n (%)	97 (56)	45 (55)	52 (58)	0.839
Comorbidities				
Hypertension, n (%)	138 (80)	66 (80)	72 (80)	0.936
Hypercholesterolemia, n (%)	133 (76)	61 (74)	72 (80)	0.380
Diabetes Mellitus, n (%)	46 (27)	22 (27)	24 (27)	0.873
Medication use				
Antiplatelet drugs, n (%)	136 (79)	59 (72)	77 (86)	*0.028*
Statins, n (%)	141 (82)	71 (87)	70 (78)	0.133
Beta-blockers, n (%)	89 (52)	40 (49)	49 (54)	0.458
ACE inhibitors, n (%)	59 (34)	26 (32)	33 (37)	0.494
Proton pump inhibitors, n (%)	87 (51)	40 (49)	47 (52)	0.652
Clinical status				
Mild ischaemia (Fontaine 1-2A), n (%)	62 (36)	25 (30)	37 (41)	
Moderate-severe ischaemia (Fontaine 2B-3-4), n (%)	110 (64)	57 (70)	53 (59)	0.143
ABPI (n=142)	0.65±0.22	0.65±0.22	0.66±0.21	0.679
ABI left rest (n=135)	0.72±0.22	0.73±0.21	0.76±0.25	0.402
ABI right rest (n=132)	0.73±0.22	0.74±0.25	0.74±0.21	0.978
Carotid IMT* (mm)	0.79 [0.15-2.78]	0.80 [0.15-2.78]	0.78 [0.35-2.06]	0.860
Carotid diameter* (mm)	7.5±1.1	7.7 [4.4-10.5]	7.3 [5.1-10.9]	0.358

Baseline characteristics of patients with PAD with coronary artery constriction (CAR constriction) or dilation (CAR dilation) during the CPT. *Indicate Mann-Whitney U test, presented as median [minimum – maximum]. P-value indicates difference between vasoconstriction versus vasodilation.

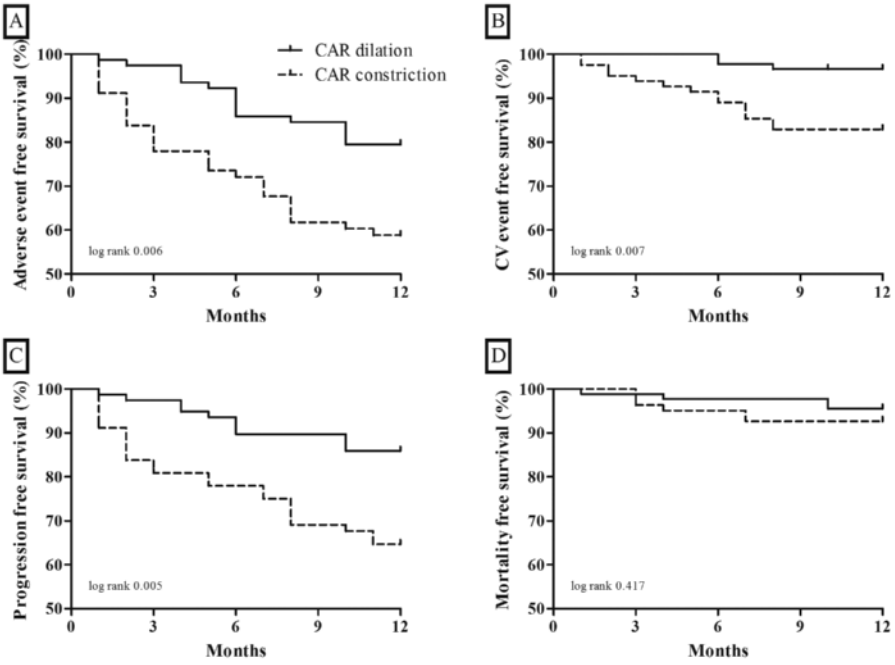

Figure 3. Kaplan-Meier survival curves for CAR

Kaplan-Meier survival curves for adverse events (A), CV events (B), clinical progression (C) and all-cause mortality (D) in PAD patients (n=172) across a 1-year follow-up. We have dichotomised PAD patients in those who demonstrate coronary constriction (CAR constriction, dotted line) or dilation during the CPT (CAR dilation, solid line). P-values relates to a Log-rank test.

Table 2. Cox analyses and hazard ratios

	Adverse events	CV events	Clinical progression	All-cause mortality
N events	70	18	61	10
Unadjusted model				
CAR dilation	Reference	Reference	Reference	Reference
CAR constriction	1.89 (1.17-3.04)	4.08 (1.34-12.41)	2.01 (1.20-3.38)	1.68 (0.47 - 5.94)
Model 1*				
CAR constriction	1.80 (1.11-2.93)	4.05 (1.33-12.36)	1.92 (1.14-3.24)	1.54 (0.43-5.60)
Model 2§				
CAR constriction	1.82 (1.12-2.95)	4.10 (1.34-12.53)	1.96 (1.16-3.31)	1.35 (0.36-5.10)

Cox analyses and hazard ratios for adverse events, CV events, clinical progression and all-cause mortality in 172 PAD patients across a 1 year follow-up. Data are presented as the hazard ratio and the 95% confidence interval (95%CI), with stepwise inclusion of a subject characteristics up to model 2. *Model adjusted for Age and Sex. § Model adjusted for Age, Sex, WHR and BMI.

Added value of clinical measures. In 30 subjects, we were unable to perform a valid ABPI because of non-compressible arteries or amputation. We were unable to perform analysis of cIMT in 3 participants because of technical problems. Analyses for cIMT (n=169) and ABPI (n=142) showed no significant effect using the Kaplan-Meier survival analysis for adverse events (Figure 4), CV events (Log-rank *P*=0.674 and 0.457, respectively), clinical progression (Log-rank *P*=0.484 and 0.153, respectively) or all-cause mortality (Log-rank *P*=0.198 and 0.795, respectively).

The cox proportional hazard models for the carotid constriction (including models 1 and 2) were repeated for the subgroups with data on cIMT and ABPI. Adding cIMT or ABPI to the fully adjusted model did not alter the HR of carotid constriction for future CV events, clinical progression, all-cause mortality or adverse events (*Supplemental material 2*).

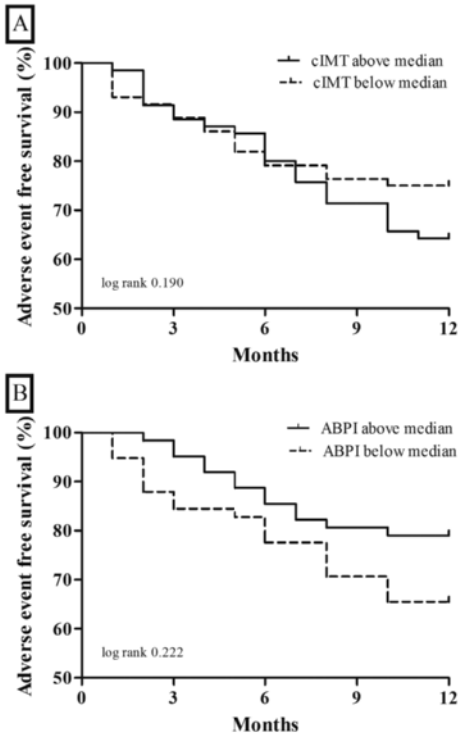

Figure 4. Kaplan-Meier survival curves for cIMT and ABPI
Kaplan-Meier survival curves for cIMT (A, 169 PAD patients), and ABPI (B, 142 PAD patients) related to occurrence of adverse events across a 1-year follow-up. The solid line represents the cIMT and ABPI above the median, the dotted line refers to the cIMT and ABPI below the median. P-values relates to a Log-rank test.

Discussion

This is the first study, to our knowledge, to examine the relation between the carotid response to the cold pressor test and future CV events in PAD patients. We found that patients who demonstrated carotid constriction during the cold pressor test had a 4.1-, 2.0- and 1.8-times increased risk at 1-year of developing a CV event, clinical deterioration and other adverse events, respectively, compared to those with carotid dilation. Importantly, the ability of the carotid vasomotor response to predict CV events was independent of subject characteristics and more predictive than other common clinical measures such as ABPI and cIMT. This suggests that a measure of (generalised) vascular health is more important than the extent of the (localised) atherosclerotic lesion in PAD patients. Therefore, a simple and non-invasive measure of carotid artery endothelial function can identify PAD patients at increased risk for future adverse CV events and clinical progression.

Dilation versus Constriction

We[14, 27] and others[13, 15, 16] have demonstrated that the CPT produces a gradual dilator or constrictor response. Similarly, normal coronary arteries show an endothelium-mediated dilation that is mediated by the CPT-induced catecholamine-release, which exceeds the direct constrictor effects of catecholamines on smooth muscle cells.[15, 16] However, endothelial dysfunction and/or (partial) endothelial damage impedes endothelium-mediated dilation leading to vasoconstriction.[15] Similar responses in the carotid artery probably explain the distinct dilator or constrictor responses we observed in our study. Surprisingly, other subject characteristics, co-morbidities and clinical measures did not differ between PAD patients demonstrating constriction or dilator responses. This suggests that CV risk factors do not contribute to the distinct vasomotion responses between PAD patients, and that the PAD disease state, rather than subject characteristics or CV risk factors contributes to carotid artery endothelial dysfunction.

Relation between the carotid vascular response and subsequent events

Current CV risk factors do not predict future CV events in patients with PAD.[12] This highlights the potential utility of the carotid artery vasomotor response in predicting future CV events. Others have demonstrated that brachial artery flow-mediated dilation predicts future CV events in PAD patients,[10, 11] but this technique is more difficult than our approach of simply measuring the relatively large carotid diameter (i.e. ~7.5 mm) in response to the CPT. Previous studies have also not measured clinical progression, whereas we found that carotid constriction had a 2-fold increased risk for loss of patency, endovascular reconstructive surgery and/or worsening in Fontaine-classification. This knowledge may allow clinicians to treat more aggressively in those patients at risk for clinical deterioration.

Others have found that the coronary artery response to CPT predicts future CV events.[17, 18] Results from the present study suggest that the carotid artery response to the CPT is a surrogate for coronary arteries. This is supported by our previous within-subject observation that a subject's carotid and coronary response to the CPT are similar.[14, 27] This observation also supports the concept that atherosclerosis is a whole body, generalised disease of the endothelium, such that an abnormal response in one vascular bed is likely also present other vascular beds. Consistent with this concept is the observation that abnormal brachial artery dilator responses to endothelial stimulation using increased flow[6] or acetylcholine[28] are associated with abnormal coronary artery responses to endothelial stimulation.

ABPI is useful in both the diagnosis of PAD29 and predicting the need for revascularisation.[2, 30, 31] Examining cIMT has also been useful in predicting atherosclerotic risk in some, but not all, studies of the general population.[24, 32] We found no relation between either ABPI or cIMT and future CV events, clinical progression or adverse events. Furthermore, adding

these clinical measures to the statistical model did not alter the relationship between carotid artery reactivity and future (CV) events. The finding that ABPI and cIMT are not related to future events contrasts with previous work performed in the general population,[2, 30, 31] but is largely in agreement with studies performed in PAD.[12, 33] Abnormal ABPI and cIMT indicate the presence of atherosclerosis, whereas coronary responses to CPT reflect endothelial function. This suggests that in individuals with known CVD, the impact of the atherosclerotic process on the endothelium is more important than the atherosclerotic lesion in predicting CV events.

A potential limitation of our study is that we did not include other biomarkers, such as high-sensitive C-reactive protein, which are demonstrated to have potential prognostic value in PAD for future CV events.[37, 38] Unfortunately, we did not assess these biomarkers to examine the potential added value of combining these markers with the CAR, which may be relevant since these biomarkers may provide additional information to our in vivo measure of endothelial function.

Clinical relevance. The carotid artery reactivity to CPT procedure is easy to perform, low-cost, non-invasive, and requires minimal time and equipment. The simplicity of the test is supported by an excellent reproducibility.[14] Moreover, in contrast to the majority of subject and/or disease characteristics, the carotid artery vasomotor response to CPT identified subjects with an increased risk for future events. The clinical relevance of the CAR may relate the identification of PAD patients who are more vulnerable to non-adherence and/or complications during surgery. For example, although symptomatic PAD should all be on drug therapy, compliance to therapy is relatively poor.[34-36] The CAR-test may help to identify individuals in whom it is of special importance to maintain compliance to drug therapy. Additionally, cardiovascular co-morbidity in PAD patients is associated with increased perioperative cardiovascular risk. Future studies are needed to assess the potential added value of the CAR to estimate perioperative risk in PAD patients.

In conclusion, our study provides the first evidence that carotid artery reactivity, independent of subject characteristics and clinical measures such as cIMT and ABPI, predicts future adverse (CV) events and clinical progression in PAD patients. The presence of carotid artery constriction during the CPT is associated with a 4-fold increased risk for future CV events and 2-fold increased risk for clinical progression. These observations suggest that the carotid artery reactivity should be further evaluated for its ability to predict future risk in patients with PAD.

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Supplemental material

Supplemental table 1. Baseline characteristics of patients with PAD, with and without a cardiovascular event during the 12-month follow-up.*Indicate Mann-Whitney U test, presented as median [minimum – maximum]. P-value indicates difference between no events *versus* events.

	Total group	**1 year follow-up**		
		No adverse events	**Adverse events**	**P-value**
Subject characteristics	n=172	n=102	n=70	
Age, y	68±10	67±10	70±10	0.056
Sex, males (%)	115 (67)	66 (65)	49 (70)	0.469
Height (m)	1.73±0.09	1.73±0.10	1.72±0.10	0.593
Weight (kg)	79.8±14.6	80.4±13.7	79.1±15.9	0.582
Body-mass index (kg/m2)	27±4	27 [17 – 39]	26 [17 – 40]	0.416*
Waist-to-hip ratio	1.014±0.098	1.00±0.10	1.03±0.10	0.067
Smoking, yes n (%)	55 (32)	36 (35)	19 (27)	
History n (%)	97 (56)	54 (53)	43 (61)	0.497
Comobidities				
Hypertension, n (%)	138 (80)	82 (80)	56 (80)	0.949
Hypercholesterolemia, n (%)	133 (80)	73 (72)	60 (86)	*0.030*
Diabetes Mellitus, n (%)	46 (27)	28 (27)	18 (26)	0.350
Medication use				
Antiplatelet drugs, n (%)	136 (79)	82 (80)	54 (77)	0.607
Statins, n (%)	141 (82)	83 (81)	58 (83)	0.803
Beta-blockers, n (%)	89 (52)	55 (54)	34 (49)	0.490
ACE inhibitors, n (%)	59 (34)	33 (32)	26 (37)	0.516
Proton pump inhibitors, n (%)	87 (51)	51 (50)	36 (51)	0.854
Clinical status				
Fontaine 1-2A, n (%)	62 (36)	52 (84)	10 (16)	
Fontaine 2B-3-4, n (%)	110 (64)	50 (45)	60 (55)	*<0.001*
ABPI (n=99)	0.65±0.2	0.68±0.21	0.60±0.18	0.068
ABI left rest (n=92)	0.72±0.22	0.75±0.21	0.68±0.23	0.572
ABI right rest (n=93)	0.73±0.22	0.75±0.23	0.69±0.21	0.335
Carotid IMT (mm)	0.79 [0.15-2.78]	0.78 [0.35–2.78]	0.81 [0.15–2.06]	0.315*
Carotid diameter (mm)	7.5±1.1	7.5±1.1	7.6±1.1	0.648
CAR%	0.8 [-19.3 - 11.6]	1.3 [-9.5 – 11.6]	-1.2 [-19.3 – 9.6]	0.068*
Constriction/Dilation (% constriction)	82/90 (48%)	40/62 (39%)	42/28 (60%)	*0.007*

Supplemental table 2. Cox analyses and hazard ratios for adverse events, CV events, clinical progression and all-cause mortality in 169 PAD patients across a 1 year follow-up. Data are presented as the hazard ratio and the 95% confidence interval (95%CI), with stepwise inclusion of subject characteristics, with the inclusion of cIMT in model 3.

	Adverse events	**CV events**	**Clinical progression**	**All-cause mortality**
N events	70	18	61	10
Unadjusted model				
CAR dilation	Reference	Reference	Reference	Reference
CAR constriction	1.92 (1.19-3.11)	4.15 (1.37-12.60)	2.05 (1.22-3.44)	1.70 (0.48 – 6.03)
Model 1*				
CAR constriction	1.84 (1.13-2.99)	4.10 (1.34-12.49)	1.95 (1.16-3.30)	1.52 (0.42-5.49)
Model 2†				
CAR constriction	1.85 (1.14-3.00)	4.13 (1.35-12.62)	1.99 (1.18-3.36)	1.36 (0.36-5.10)
Model 3‡				
CAR constriction	1.85 (1.14-3.00)	4.14 (1.35-12.67)	1.99 (1.18-3.36)	1.47 (0.39-5.52)

*Model adjusted for Age and Sex. † Model adjusted for Age, Sex, WHR and BMI. ‡ Model adjusted for Age, sex, WHR, BMI and cIMT.

Supplemental table 3. Cox analyses and hazard ratios for adverse events, CV events, clinical progression and all-cause mortality in 142 PAD patients across a 1 year follow-up. Data are presented as the hazard ratio and the 95% confidence interval (95%CI), with stepwise inclusion of subject characteristics, with the inclusion of ABPI in model 3.

	Adverse events	**CV events**	**Clinical progression**	**All-cause mortality**
N events	54	12	45	9
Unadjusted model				
CAR dilation	Reference	Reference	Reference	Reference
CAR constriction	1.84 (1.07-3.16)	2.52 (0.76-8.38)	1.98 (1.09-3.60)	1.55 (0.42-5.77)
Model 1*				
CAR constriction	1.78 (1.02-3.08)	2.31 (0.69-7.74)	1.88 (1.02-3.44)	1.54 (0.39-6.09)
Model 2†				
CAR constriction	1.78 (1.03-3.09)	2.29 (0.68-7.78)	1.90 (1.04-3.47)	1.08 (0.24-4.81)
Model 3‡				
CAR constriction	1.78 (1.03-3.08)	2.32 (0.68-7.86)	1.89 (1.03-3.46)	1.01 (0.22-4.54)

*Model adjusted for Age and Sex. † Model adjusted for Age, Sex, WHR and BMI. ‡ Model adjusted for Age, sex, WHR, BMI, and ABPI.

Part 3

Chapter 7

General discussion

The general aim of this thesis was to improve performance of the "older" measures of endothelial function and to explore "newer" indices. In this chapter, the findings of the preceding individual chapters are summarized and discussed in relation to the contemporary literature. Finally, I provide a future perspective and highlight remaining knowledge gaps surrounding the assessment of endothelial function in humans.

Flow-mediated dilation guidelines, a walk through time

Part I of my thesis deals with the "older", traditional measure of endothelial function, the flow-mediated dilation (FMD). The origin and development of FMD dates back to 1992 and the milestone study of Celemajer *et al.* that introduced assessment of conduit artery diameter responses to an increase in blood flow, induced by 5-minute cuff occlusion (Figure 1).[1] Based on the promising results from initial studies, its apparent simplicity and low costs, the number of studies adopting the FMD methodology rapidly multiplied. Studies demonstrated that FMD may be used as a surrogate measure of coronary endothelial function.[2, 3] Others explored the potential prognostic value of FMD in different populations.[4-12] Unfortunately, many follow-up studies deviated from the original protocol, which contributed to significant between-study differences in methodology. Indeed, a meta-analysis from 2005 acknowledged the presence of wide variation in baseline FMD between studies, with resting FMD between 0.2% to 19.2% in healthy individuals, and -1.3% to 15% in diseased individuals.[13] Several technical aspects were identified that may contribute to the variation between studies, such as the occlusion site and occlusion duration. Variation in the protocol may contribute, at least partly, to the heterogeneity in baseline FMD values. It was suggested that uniform methodology would facilitate more reliable between-study comparisons.[13]

Although Corretti published their guidelines in 2002,[14] these guidelines left many questions unanswered. The most recent guidelines date from 2011, when Thijssen *et al*. collaborated with international groups to improve the practical guidance and technical approaches for valid FMD assessment.[15] These guidelines focussed on the technical issues involving duplex ultrasound, measurement methodology (i.e. continuous assessment of at least 180sec post-deflation, and relevance of baseline diameter), and data analyses (i.e. using edge-detection software, and shear normalisation). Nonetheless, despite using these guidelines, variation within and between studies remains (**Chapter 2** and **Chapter 3**). This raises the question about the utility of the FMD as a valuable tool for (clinical) assessment of vascular function.

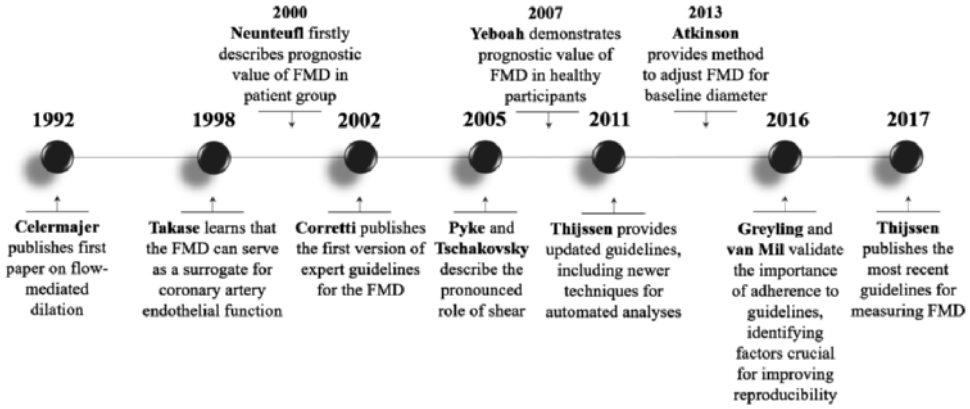

Figure 1. FMD timeline
A timeline of the development of the flow mediated dilation technique, including key papers and discoveries.

FMD, clinical utility, research tool, or both?

In 2010, the American College of Cardiology (ACC) and the American Heart Association (AHA) published new guidelines for the assessment of cardiovascular risk in asymptomatic adults, including discussion of various strategies and tests (e.g. carotid intima-media thickness, pulse-wave velocity).[16] Their classification of recommendations and the level of evidence is provided in Figure 2.

The ACC-AHA taskgroup ranked the evidence available for specific methodologies and tests to either the level A (multiple randomized controlled trials and/or meta-analyses published), level B (single randomized trial or non-randomized studies), or level C (limited literature available, based primarily on consensus opinion of experts). Additionally, methods are graded on their proven benefit, which resulted in a final recommendation (Figure 2). Their recommendation was that FMD is not recommended for cardiovascular risk assessment in asymptomatic adults (Class III, no benefit, evidence level B).

Their recommendation that measuring FMD was not beneficial was based on 2 reasons; *1)* technical difficuluties resulting in considerable variability between studies, and *2)* the modest evidence regarding the prognostic value in asymptomatic adults and when controlling for traditional risk factors. Below, I have discussed both reasons, taking our own findings into consideration.

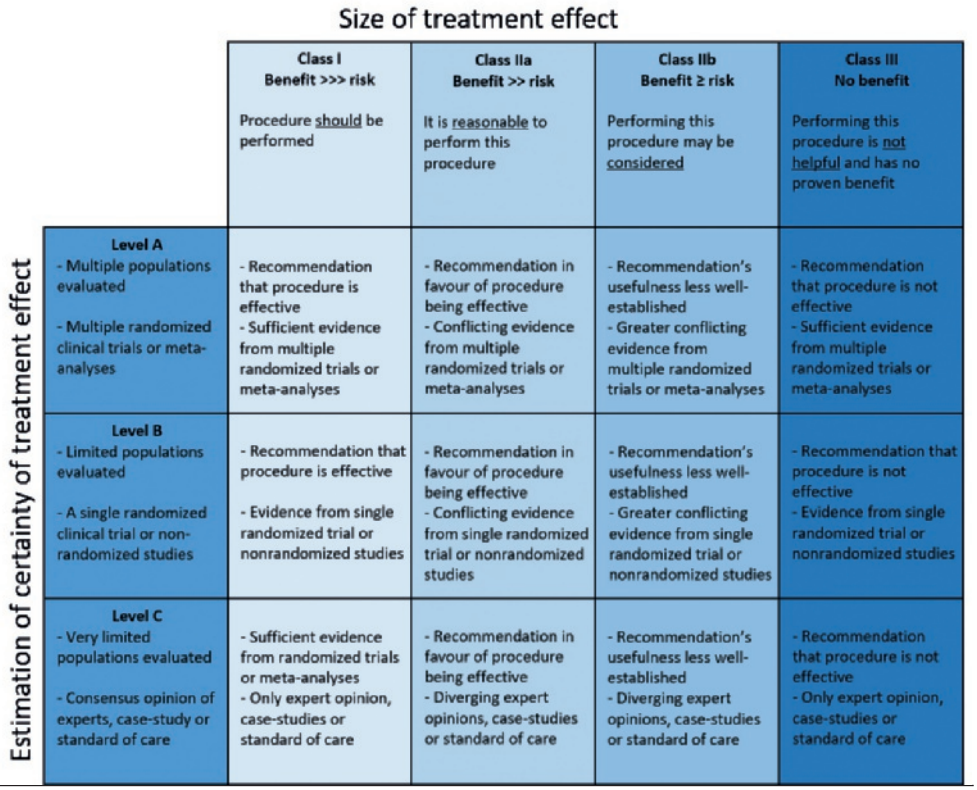

Figure 2. ACC-AHA recommendations
Adapted from the ACC-AHA guidelines 2014, Greenland et al.[16]

Technical difficuluties and between-study variability; what can we do?

In this thesis we identified several technical factors that independently contribute to FMD variability, including baseline diameter, operator experience, and use of a stereotactic probe holder. These factors will be further considered below, and I will provide recommendations to improve future FMD assessment.

Baseline diameter. FMD is negatively associated with the baseline diameter, meaning that a smaller baseline diameter likely leads to a larger FMD, and *vice versa*.[1, 17, 18] Studies involving between-group comparisons (e.g. healthy *versus* diseased) are urged to report their absolute diameter changes, to account for between-subject variability.[19-21] Between-subject comparisons are more susceptible to differences in baseline diameter and incorrect assessment of the true endothelial function. This emphasises the importance of normalising the FMD for baseline diameter.[11, 12, 17] A possible method of normalising FMD for baseline diameter can be achieved by allometric scaling, which accounts for the absolute diameter

changes in respect to the logarithmic/linear slope between peak and baseline diameter.[17] Within-subject comparisons are easier to perform, as baseline diameter is comparable. However, in the case of longer time lapses between repeated measurements, biological variability (aging) and environmental factors (e.g. exercise training results in arterial remodelling, reducing initial functional improvement back to baseline),[22-24] differences in baseline diameter need to be accounted for by scaling accordingly.

Level of experience. To our knowledge, our work (presented in **Chapter 2**) represents the first evidence that laboratory experience importantly affects reproducibility of the FMD. Despite the deceptively simple appearance, FMD assessment is technically challenging and requires proper training before active data collection.[14] Nonetheless, qualifying 'proper' training remains difficult. Since 'sufficient level of training' is a sensible path to follow when performing FMD work, studies often present a brief statement regarding involvement of experienced sonographers without further specifications of operator training and/or reproducibility.[19, 25, 26] Previous described specific instructions included hands-on training by an experienced individual, and making at least 100 scans annually to maintain competency.[14] Another study choose to incorporate a formal training programme; one was eligible to scan when 10 repeat scans were performed with <2% variability.[27] Others followed with a similar training protocol, consisting of 20 supervised scans, resulting in a quality certification following 5 consecutive scans with variation <2%.[28] Due to a steep learning curve in the FMD assessment, some talented operators will obtain the desired level within a short period of time (e.g. 2-4 months), whilst others require a more extensive period (up to 6 months and longer). This should be taken into consideration when designing a new study.
Since previous FMD training and operator experience is seldom reported (**Chapter 3**; 307 of 376 publications did not report reproducibility), extensive disclosure of operator- and analyst training and reproducibility is vital. We recommend reporting the inter-operator coefficient of variation. When this is <2% variation within repeated measurements, the operator is considered skilled enough to validly assess FMD. Additionally, we advise the ability to perform repeated scans (e.g. 5 consecutive scans) with <2% variation at any given moment, maintaining the desired level of scanning.

Probe holder versus handheld. The use of a probe-holder has been a point of discussion since the beginning of the FMD era.[14, 15] Beux *et al.* described that using a probe-holder improved FMD reproducibility, as the holder allows for fine adjustments through small movements (using micrometer screws).[29] These findings were supported by others, demonstrating that using predefined settings for a stereotactic probe holder is feasible and yields reproducible results.[19, 28] None of these studies, however, provided evidence that using a probe-holder results in better reproducibility compared to the hand-held approach. Findings from **Chapter 3** indicated that use of a probe holder was associated with lower FMD variation between

studies. Nonetheless, in line with our findings from **Chapter 2** and others,[30] it is possible that highly skilled operators are able to conduct FMD measurements with high reproducibility, regardless of the use of a probe holder. The discussion concerning superiority of the method is still undecided. Future studies are warranted to directly compare the stereotactic probe-holders with the hand-held approach.

To briefly summarize, the factors mentioned above independently contribute to the wide variation between studies. To improve FMD reproducibility, I recommend *1)* reporting absolute baseline diameter (and scaled accordingly when differences between groups occur), *2)* describing operator training and experience (preferably as coefficient of variation), and *3)* direct comparison between hand-held and probe-holder use to determine superiority. Combining these recommendations with adherence to expert-consensus guidelines will reduce between-study variability.

Adherence to the guidelines and the prognostic value

Expert-consensus guidelines have contributed tremendously to the standardization of technical approaches and set minimum standard requirements for FMD measurements.[14, 15, 31] However, this thesis described large variation in the adherence to guidelines between studies (2.4 to 9.2, **Chapter 3**), whilst almost every paper refers to using these guidelines. In Table 1 we take a closer look into several prognostic FMD studies, specifically exploring how well these studies adhered to FMD guidelines using the previously described scoring tool (**Chapter 3**). Interestingly, the average score of the studies in Table 1 is 3.4 points, and varies between 0 and 5.6 out of 10. In other words, none of the studies examining the prognostic value of the FMD for future CV events meet the minimum required level of adherence of 6 (since studies with adherence >6 showed acceptable-to-good reproducibility in **Chapter 3**).

Although most of these studies were published before the latest revision of the methodological guidelines by Thijssen *et al.*, it is striking that even recent studies do not comply to these guidelines. For example, a recent study describing predictors of endothelial dysfunction in patients with rheumatoid arthritis, measured FMD with upper arm occlusion.[21] Moreover, a detailed description of the methodology is absent, leading to an adherence score of only 0.4. The FMD for their healthy control group is ~11±0.5%, and 6±2% in patients with rheumatoid arthritis. These values are remarkably high, especially when comparing these with our previous analysis of 109 healthy participants which resulted in a FMD of 6.6±2.8%, meaning that only 10% of our population had an FMD of 11% or higher.

The potential impact of not following the FMD guidelines becomes apparent from another recent example, where FMD was assessed in non-alcoholic liver disease in postmenopausal women.[20] Whilst this study reported adherence to the published guidelines, detailed inspection revealed that the researchers do not clearly describe the included control group, and details on operator training are missing, leading to an adherence score of 3.6. This study

demonstrated a remarkably high resting FMD of ~15% in the healthy control group. Based on our previous work and the work performed in **Chapter 3**, this again seems highly remarkable and may be caused by not following the guidelines.

Table 1. FMD studies and their scores

First Author	Year	N	Age	Group	FMD%	Baseline diameter	Score
Patient population							
Neunteufl	2000	73	51	Chest pain	5.7±4.8%	4.0±0.7	2.4
Gokce	2003	199	67	PAD	6.6±4.7%	3.9±0.7	2
Brevetti	2003	131	64	PAD	7.6% [5.3; 9.9]		2.2
Fathi	2004	444	58	CAD	5.2±6.1%	3.3	2.6
Frick	2005	398	54	Chest pain	7.6%	4.3	2.4
Patti	2005	136	63	CAD	7.0%		4
Kitta	2005	141	65	PCI	3.5-7%		1.6
Suzuki	2008	377	67	MS	5.26±3.7		4
Skjold Ulriksen	2009	223	54	Chest pain	4.7±2.7%		2.6
Tarro Genta	2013	71	65	HF	6.5±4.0%	3.7±0.7	5
Pugh	2014	34	48	NAFLD	4.8% [4.1;5.4]	4.2 [3.9;4.6]	7.4
Paine	2016	156	56	HF	4.6±3.4%		5.6
Kubo	2016	80	69	Chest pain	4.2%		3.8
Hamid	2016	78	54	MI	5.9±3.1%		0
Healthy population							
Yeboah	2007	2792	79	asymptomatic	2.7%	4.5±0.9	5.4
Rossi	2008	2264	54	asymptomatic	6.3%	3.9±0.6	3.4
Shechter	2009	435	54	asymptomatic	11.7±3.2%	5.8 ± 0.9	6.6
Yeboah	2009	3026	61	asymptomatic	4.4±2.8%	4.3±0.8	5.4
Lind	2011	921	70	asymptomatic	4.7±3.6%		4.0
Shechter	2014	618	55	asymptomatic	12.0±3.2%	5.7±1.0	5.8

Prognostic value of the FMD, in patient- and healthy populations. Adapted from Green et al 2011. CAD; coronary arterial disease, PAD; peripheral arterial disease, CAG; coronary angiography, MS; metabolic syndrome, PCI; percutaneous coronary intervention, HF; heart failure, NAFLD; non-alcoholic fatty liver disease, MI; myocardial infarction. Either expressed as mean ± standard deviation, or median [interquartile range].[4-6, 8, 10-12, 32-44]

Regardless of the various expert-consensus guidelines, many current studies do not comply with these guidelines.[14, 15, 31, 45] This contributes to questionable FMD outcomes, and leads to high variability within and between studies. Therefore, I believe that studies with poor adherence scores (i.e. <6, and application of continuous diameter recording, edge-detection

and wall-tracking software is mandatory) should not be published and/or taken into consideration in (meta-analytical) reviews, as the risk of measurement error and over/under sampling will result in ambiguous findings and more between-study variability. Our newly proposed scoring tool to assess the level of adherence (**Chapter 3B**) could serve as a guide to aid researchers in standardizing the FMD approach. Preferably, authors should report their individual adherence score to the guidelines using the tool we published previously, or improve their applied methodology and publication accordingly.

We also observed significant variation in FMD between studies in Table 1, even when comparing similar patient populations. For example, the study from Neunteufl *et al.* reported a FMD of 12.6±6.7%,[8] whilst Kubo *et al.* reported a FMD of 4.2%,[35] both in patients with chest pain. The substantial disparity in FMD is also visable when comparing asymptomatic participants with clinical groups. For instance, our previous analysis of 109 healthy participants resulted in a FMD of 6.6±2.8%, which is remarkably lower than reported by the study from Brevetti *et al.* in patients with peripheral arterial disease (average FMD of 7.6%).[4] This implies that any FMD value could refer to supra-normal, normal or sub-normal values, dependent on the laboratory and protocol that was followed. The construction of reference values (and following current guidelines) could assist in the more accurate interpretation of FMD, thus lessening the between-study variability.

Flow mediated dilation, lessons learned

In summary, I believe we have reached a turning point in the research involving the "old" FMD technique. To meet the AHA-ACC required guidelines for assessment of CV risk, we should adhere to the above-mentioned recommendations and expert-consensus guidelines, achieving at least a score of 6 out of 10 related to the adherence score. Studies with poorer adherence should not be taken into consideration when making evidence-based conclusions based on literature searches. This will mitigate the variation between and within studies. Moreover, this also relates to the FMD's true prognostic value in clinical populations. Additionally, reference values for the FMD (when adopting the guidelines) are necessary for correct interpretation of an FMD value. Having these reference values for several populations (i.e. young *versus* old, healthy *versus* disease), will positively contribute to reducing measurement variability.

Carotid artery reactivity, a new step forward?

The second part of this thesis focusses on the development and validation of a newly introduced test of vascular health; the carotid artery reactivity (CAR) test. Given the novelty of the CAR and lack of studies exploring the mechanisms for carotid dilation during sympathetic nervous system activity, many questions regarding the physiological mechanism are currently unanswered. However, similarity in responsiveness between the carotid and coronary arteries was previously suggested.[46] Therefore, to provide an initial overview on

the potential mechanisms underlying carotid dilation during sympathetic activity, I will first discuss studies that provided mechanistic insights into coronary artery responsiveness to sympathetic stimulation. Specific information on carotid artery reactivity is provided when available. Whether all assumptions on coronary reactivity can be applied to the carotid artery is currently unknown and warrants further evaluation.

Coronary arteries and sympathetic stimulation. The coronary circulation is highly responsive to sympathetic stimulation, such as demonstrated by Folkow *et al*., who reported the innervation of the coronary vasculature by sympathetic adrenergic fibres (Figure 3).[47, 48] In a subsequent study, Feigl demonstrated that generalized sympathetic stimulation results in an increase in myocardial blood flow.[49, 50] Several mechanisms seem to contribute to this increase in blood flow, including an increase in metabolic demand, changes in flow, and neuro-hormonal stimuli.[51-53] Importantly, the involvement of these mechanisms to coronary vasodilation seem to relate to immediate sympathetic stimulation (i.e. following a stress-response). A known test to provoke sympathetic activity, is the cold pressor test (CPT).[54] CPT increases catecholamine release[55] and coronary flow, which can be mediated by a local increased metabolic demand, blood flow (or shear stress)-mediated dilation and/or neural factors.[52, 56-59] The neural component refers to an adrenergic-mediated vasoconstriction, to maintain a uniform transmural blood flow distribution in the cardiac muscle.[49, 50] The metabolic, flow-mediated and neural vasoactive responses together mediate a vasodilator response of coronary arteries, whilst current work is unable to untangle the exact contribution of these various vasoactive pathways that closely work together.
Interestingly, a study in 1986 found that removal of the coronary endothelium in dogs resulted in abolished vasodilation responses to sympathetic provocations (infusion with norepinephrine), whilst intact endothelium resulted in pronounced vasodilation.[60, 61] This implies, to some extent, a role for the endothelium. The importance of functionally intact endothelium was further verified by multiple studies. For example, it was shown that infusion of acetylcholine, a strong endothelium-dependent vasodilator, mirrored the effect of sympathetic stimulation; vasodilation in intact coronary arteries.[56, 58, 62, 63] Schächinger *et al.* examined whether responses to both acetylcholine and CPT could independently predict the occurrence of future cardiovascular events and disease in CV patients.[57] Later also confirmed by others, Schächinger found that coronary vasoreactivity measured with both acetylcholine and CPT, could predict long-term atherosclerotic disease progression and cardiovascular events.[57, 64, 65]

Carotid arteries and sympathetic stimulation. When examining peripheral arteries as a potential surrogate for the coronary circulation, sympathetic activation does not necessarily lead to similar peripheral artery responses as observed in coronary arteries.[59, 66-69] More specifically, previous work found no change or a small constriction of peripheral arteries upon

sympathetic stimulation. However, a previous observational study found that the carotid artery shows marked dilatation in response to CPT.[46] This implied similarity between carotid and coronary artery responses to CPT, supposedly the result of the balance between adrenergic vasoconstriction and endothelium-mediated vasodilation.[46] In this thesis, we followed up on this observation and confirmed similarity in responses between the carotid and coronary arteries (**Chapter 4**). These studies suggest that the carotid artery shows arterial responses that reflect those observed in the coronary circulation. Given the easy access of the carotid artery, this raises several questions about the potential use of this physiological phenomenon. Considering the previous established AHA-ACC guidelines,[16] a test for cardiovascular health assessment should *1)* have a strong empirical literature background, *2)* requires a technical achievable methodology, and *3)* be beneficial for risk assessment independent of risk factors. These AHA-ACC criteria are further discussed below in relation to the CAR.

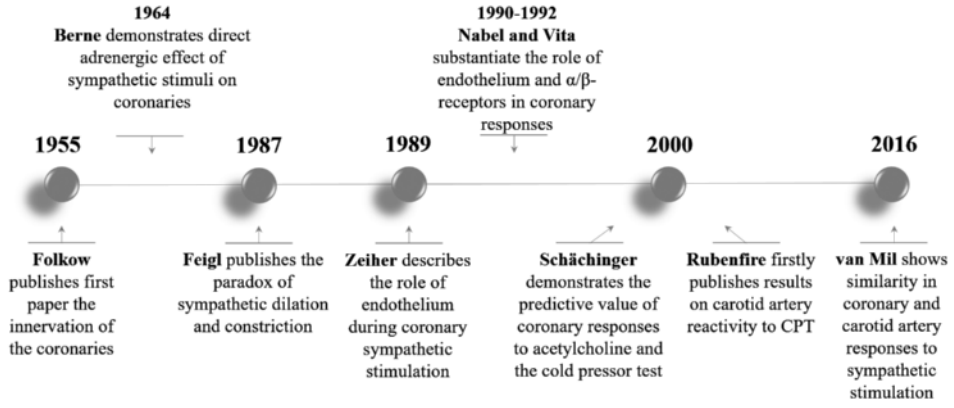

Figure 3. CAR timeline
A timeline of the development of measuring coronary and carotid reactivity to sympathetic stimulation, including key papers and discoveries.

Strong empirical background

Similarity in responses between the coronaries and carotid. Ideally, a test of vascular function that predicts coronary events should reflect the vascular health of the coronary arteries, which are particularly susceptible for endothelial dysfunction and atherosclerosis. The carotid shows similar responsiveness as the coronary artery responses to CPT.[46] When we combine data from **Chapters 4** and **5**, we have obtained measurements of coronary artery function in 49 participants. These include measures from both the CPT test and the lower body negative pressure test (LBNP). We found a significant correlation of 0.59 for CPT and LBNP results combined (Figure 4). This further strengthens our earlier findings (**Chapters 4** & **5**), demonstrating similarity between carotid responses and coronary flow responses to sympathetic stimulation. Moreover, these findings suggest the CAR may serve as a surrogate measure of coronary vascular health.

An important limitation of these previous observations must be discussed. Whilst measuring carotid artery diameter and velocity is feasible, measuring coronary velocity *and* diameter with non-invasive echo Doppler techniques is currently technically not feasible. However, coronary flow velocity is documented to reliably reflect changes in absolute coronary blood flow. Several studies have adopted coronary flow velocity (measured as the velocity time integral, cm) as a model to non-invasively assess coronary dilation.[59, 70-72] Since this is still an indirect measure of coronary artery blood flow, we would ideally reproduce the similar responses between the carotid and coronary arteries using invasive, gold standard techniques. Such studies are recommended to better understand the CAR-test and should be done in a catheterisation laboratory, which allows for direct and simultaneous comparison of both vessel responses. Finally, it is essential that these findings are reproduced in patient populations as well.

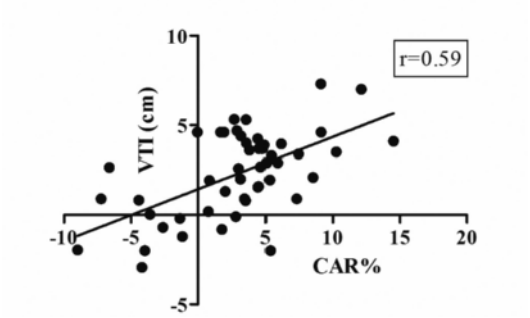

Figure 4. Correlation CAR and coronaries
Correlation (Pearson) of carotid artery reactivity, percentage dilation (CAR%) and coronary artery flow velocity in n=49 participants, expressed as the velocity time integral (VTI) in cm.

The eliciting stimulus. To further understand the empirical background of CAR, it is important to understand the underlying mechanisms contributing to the vasomotor response of the carotid artery to sympathetic stimulation. In **Chapter 4**, I discussed the potential role for shear stress and blood pressure as mediating factors for carotid vasomotor responses. The slower increase in both shear rate and blood pressure compared to the diameter change make these stimuli unlikely as the primary triggers that mediate carotid artery dilation. In addition, no relation was found between changes in blood pressure or shear stress and carotid artery diameter changes. Furthermore, despite the presence of increases in blood pressure, some participants demonstrated a decrease in carotid artery diameter. This makes it unlikely that the carotid artery diameter response represents a shear- and/or pressure-driven vasomotor response.

Metabolic demand. As discussed earlier in the Discussion, several mechanisms contribute to the sympathetically mediated increase in coronary flow. These mechanisms include an increase in local metabolic demand, endothelium mediated vasodilation, and neural mediated vasoreactivity. Berne *et al.* previously acknowledged the key role of coronary metabolic activity (i.e. myocardial oxygen consumption) in mediating coronary flow.[53, 73] Feigl reported that a significant decrease in coronary sinus oxygen was present, when changes in coronary metabolism were absent (blunted by propranolol).[74] This suggested that myocardial oxygen uptake was not only regulated by myocardial demand, but also by coronary sympathetic activity. The contribution of adrenergic receptors in mediating coronary oxygen uptake was confirmed by Mohrman *et al.* who found that α-blockade caused a decrease in coronary oxygen uptake.[75] Finally, Di Carli *et al.* examined coronary flow in transplant patients, where both denervation and regional re-innervation of cardiac segments could be studied simultaneously. It was demonstrated that increases in coronary flow in response to CPT were larger in areas in re-innervated areas, which suggests that coronary flow is regulated to larger extent by adrenergic mechanisms than via metabolic regulation. Taken together, as was also nicely summarised in a recent review from Barbato *et al*., increases in cardiac metabolism *and* coronary sympathetic vasodilation are involved in adrenergic vasodilation. Since it is challenging to study these factors independently, it remains unclear to what extent the metabolic and adrenergic components interact and contribute to coronary vasomotion during sympathetic activation.

Endothelium-mediated dilation and role of NO. The pivotal role of the endothelium in the coronary responses to sympathetic stimulation was examined by assessing coronary responses to sympathetic stimuli in healthy and atherosclerotic and/or (pharmacologically) denuded coronary segments (in animals).[56, 58, 60, 61, 63] Several studies demonstrated that both CPT and acetylcholine induced vasoconstriction in atherosclerotic coronary segments, whilst infusion with nitroglycerin (a potent endothelium-independent vasodilator) induced vasodilation. The preserved vasodilation in response to nitroglycerin in atherosclerotic arteries suggests the importance of the integrity of the endothelium.[56, 58, 63] Tousoulis *et al*. demonstrated that coronary vasodilator responses to CPT could be reduced by inhibition of NO synthase (L-NMMA). Inhibition of NO synthase not only attenuates the NO-mediated dilation, but may also potentiate adrenergic mediated constriction,[76, 77] demonstrating the strong interaction between adrenergic and endothelial control mechanisms.

α- and β-receptors. CPT produces sympathetic release of catecholamines (e.g. norepinephrine and epinephrine),[55] which are both present on the luminal (i.e. circulating) and abluminal side, that influence coronary vasomotion through stimulation of adrenergic receptors, present on both coronary endothelial and vascular smooth muscle cells. Below, the roles of the α- and β-receptors are discussed. Both α- and β adrenergic receptors are involved

in coronary vasomotion in response to activation of the sympathetic nervous system (Table 2).[51] The β-receptors induce vasodilation, which results in an increase in coronary blood flow. The α-receptors concurrently induce minor vasoconstriction to limit the metabolic demand. This is to ensure maintenance of blood flow in the inner layers of the ventricles. This results in a delicate balance between α-mediated vasoconstriction and β-mediated dilation. If the endothelium is dysfunctional, dilation evoked by sympathetic stimuli is abolished, partly due to endothelial β-receptor failure, impaired endothelium-mediated NO-release, and/or enhanced α-receptor responsiveness.[51, 56, 63, 76]

α-receptors. The α-receptors (both α-subsets, Table 2) are predominantly responsible for coronary vasoconstriction. Stimulation of α1-receptors, which are located on the vascular smooth muscle (VSM) cells, cause vasoconstriction.[51, 78, 79] In healthy subjects, infusion of α1-agonists does not influence coronary vasomotion.[80] This is due to the β-mediated dilation through the functionally intact endothelium, counterbalancing the α1-mediated constriction. In diseased participants (i.e. those with coronary stenosis), however, infusion of α1-agonist (i.e. methoxamine and phenylephrine) resulted in augmented constriction.[51, 58, 63]

Stimulation of α2-receptors on the VSM cells mediates neural-mediated constriction, whilst receptors located on the endothelium cause dilation through NO-release during sympathetic stimulation (Table 2).[51, 60, 81, 82] Selective α2-adrenergic agonists can cause endothelium-dependent relaxation in the coronary arteries of healthy subjects,[82] whilst concurrent stimulation of α2-receptors on the VSM cells demonstrated microvascular constriction.[80, 83] In contrast, intracoronary infusion of selective α2-agonists induced vasoconstriction in patients with atherosclerotic coronaries. This possibly results from a loss of endothelial α2-receptors. Normally endothelial α2-receptors contribute to luminal vasodilation, whilst VSM cell-mediated α2-receptors facilitate abluminal constriction. The net result of endothelial dysfunction could result in enhancement of VSM cell mediated α2-vasoconstriction.[80, 84-86]

β-receptors. β-receptors are predominantly present on the coronary endothelium, causing vasodilation during sympathetic activation (Table 2). There are 3 subtypes, located on either the endothelium or the VSM cells, and the vast majority corresponds with the β2-receptor subtype. In healthy participants, infusion of a β2-receptor agonist (salbutamol) induces dilation. Interestingly administration of selective β-receptor blockades in healthy participants (e.g. infusion with propranolol),[56] partly inhibited vasodilator responses to CPT. Since β2-receptors are more strongly antagonized by propranolol than β1-receptors, possibly β1-receptor activation contributed to the remaining dilation.[87] It is possible that the paradoxical constriction of coronary arteries in patient groups results from disturbed β-receptors, due to endothelial dysfunction. Indeed, intracoronary infusion of a β2-receptor agonist (salbutamol) showed an impaired vasodilator response in atherosclerotic coronary arteries.[56]

Carotid artery. In summary of the discussions above, the exact contribution of both adrenergic receptors during CPT to coronary vasoreactivity remains to be further clarified. More importantly, scientific work on the adrenergic innervation of the carotid artery is scarce

and reliant on animal work.[88] Accordingly, future studies are necessary to explore the roles of the endothelium and adrenergic innervation of the carotid artery during sympathetic activation. We saw in **Chapter 5** that administration of Prazosin, a selective α1-receptor antagonist, resulted in an attenuated carotid dilator response in healthy participants, whilst one would expect a more pronounced vasodilation. This can possibly be attributed to concurrent adrenergic α2-receptor activity on the VSM cells. These findings warrant further investigation into adrenergic activation of the carotid artery. Similarly, the precise role of the carotid endothelium needs to be investigated.

Table 2. α- and β-receptor types

Receptor	Location	Cell affinity	Role
α-receptors			
α1	larger vessels	VSM cells	Vasoconstriction
α2	microcirculation	VSM cells and endothelium	vasoconstriction and vasodilation
β-receptors			
β1	epicardial coronaries	endothelium and VSM cells	Vasodilation
β2	Arterioles	endothelium and VSM cells	Vasodilation
β3	Arterioles	endothelium	Vasodilation

Subsets of α- and β-receptor types in the coronary circulation. VSM; vascular smooth muscle

Technical achievable methodology

The AHA-ACC guidelines require a technical achievable methodology before further recommendation as a useful vascular test. Major problems with the current techniques to assess endothelial function, relate to *1)* technical difficulties, and *2)* methodological variability and adherence to guidelines.

Technical difficulties. The CAR test is in some ways analogous to FMD, as both tests use pulsed wave Doppler and ultrasound techniques to assess endothelial function. As both tests are reliant on vascular function, subject preparation (e.g. 15 minutes of supine rest, fasted state, measurements at the same time of the day) and data analyses (e.g. continuous assessment, using semi-automated edge-detection software) are performed in a similar fashion.[15] However, some of the factors that significantly influenced the FMD, are less likely to influence CAR. This supports the concept that CAR is a robust test of endothelial function. For example, baseline diameter is not related to CAR, as we already saw in **Chapter 4**. This is further supported by combining our data in healthy participants (n=189, r=0.12, *P*>0.05). This indicates that CAR is independent of measures of carotid size, and between-study variability is not influenced by baseline diameter. Second, CAR is easy to perform, as the carotid is a larger and more superficial artery, making the operator training less tedious. We demonstrated a within-day

coefficient of variation (CV) of 2.6% and between-day CV of 2.8% for CAR in **Chapter 4**. This indicates the CAR is highly reproducible.

Methodological variability. When assessing **Chapters 4** and **5**, CAR methodology is corresponding very well with the adherence scoring tool for FMD, scoring a 9.2 on both papers. Differences in the methodology (e.g. time immersed, temperature of the water, **Chapter 4** and **5**) are to be incorporated in guidelines to assure for uniform methodology between studies. Therefore, I recommend that when performing CAR tests, adherence to the expert-consensus guidelines for peripheral vascular assessment as described in this thesis is necessary, to keep methodological variability at a minimum. Factors contributing to CAR variation (similar to **Chapter 2**) can be explored to optimise the methodology. Accordingly, the methodology described in our **Chapters 4** and **5** need to be incorporated to provide a similar expert-consensus guidelines and scoring tool for the CAR test specifically.

Risk assessment and risk factors, the prognostic value of CAR

Risk factors. The key prerequisite of the AHA-ACC guidelines for a new test to assess cardiovascular health, is that the test needs to be beneficial for risk assessment. A series of longitudinal studies, preferably from various laboratories, is required to obtain detailed insight. Obviously, this is currently not available and we will discuss the limited evidence and experiences from our laboratory. First, we explored whether CAR relates to the presence of established cardiovascular disease risk factors. We showed in **Chapter 4** that older participants had a significant lower CAR% than their younger peers. We also found that participants with more cardiovascular risk factors had a lower CAR%. To further substantiate these latter findings, we performed a study in 85 healthy octogenarians (average CAR% 2.9±2) who participated in the Nijmegen Four Days Marches of 2016. When assessing the relation between risk factors and CAR, we found no differences in CAR between those with 0, 1 and ≥2 risk factors (2.7±1.7, 3.3±2.1, and 2.4±1.5, respectively, P=0.183). This is not in line with our previous findings. Possibly, this finding can be explained by the group we included. Previous work found that, although CVD prevalence does not markedly attenuate in octogenarians, traditional risk factors do not identify those who will develop future CV events.[89] This may contribute to the observation that CAR does not relate to CV risk factors in this population.
Average CAR in the octogenarians was relatively high (i.e. 2.9%) and even higher than the healthy older group from **Chapter 4** (i.e. 1.8±2.6%). Moreover, no vasoconstriction was reported in these healthy octogenarians, whilst 25% of the healthy older group from **Chapter 4** reported constriction. A possible explanation is that our octogenarians were physically active, since they all participated in the four days marches, and therefore demonstrated favourable vascular health. To support the possible relation between level of physical fitness and CAR, preliminary data from our laboratory in 18 participants with increased CVD risk showed that 12-weeks of exercise training significantly improved CAR (data not presented). Moreover, six

patients who showed vasoconstriction prior to training were reversed to vasodilation after exercise training. This implies that CAR can be improved by exercise training, with a (larger) dilation being related to improved physical fitness.

Intima media thickness. A known risk factor and independent predictor of CV events is the carotid intima-media thickness (cIMT). The cIMT is a frequently used clinical test,[90, 91] classified as an IIa class test by the AHA-ACC guidelines,[16] whilst its clinical value is still being debated.[91-93] Since both CAR and cIMT relate to the carotid artery, it seems sensible that the CAR should exceed the prognostic and discriminative value of the carotid artery wall thickness. We saw in **Chapter 4** that CAR was not related with cIMT in healthy participants. When all data from **Chapters 4-5-6** as well as additional testing in the octogenarians on CAR and cIMT were combined (n=296), we still found no association between CAR and cIMT (r=0.04, *P*>0.05, Figure 5). Moreover, findings of **Chapter 6** demonstrated that carotid artery wall thickness did not predict outcomes or contributed to the hazard models for the CAR. A logical explanation for the lack of relation, despite that both tests involve the carotid artery, is that CAR examines functional characteristics and cIMT addresses structural aspects. Therefore, CAR provides novel, additional information since information derived from CAR seems independent of carotid artery structure. This seems the case for both asymptomatic participants and patients with cardiovascular disease. These observations highlight the potential (clinical) relevance of measuring both CAR and cIMT.

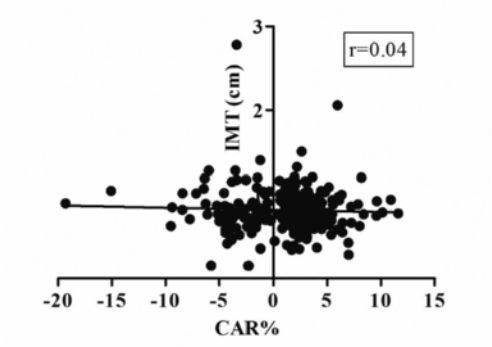

Figure 5. Correlation CAR and cIMT
Carotid artery reactivity, percentage dilation (CAR%) versus the intima-media thickness (cIMT) in n=296 participants, demonstrating a non-significant correlation (P>0.05).

Prognostic value. Ideally, the CAR test would be able to predict future cardiovascular events and disease, independent of traditional risk factors. In **Chapter 6**, we found that PAD patients with a vasoconstrictive response to the CPT had a 4-fold higher risk for future CV events, 2-fold increased risk for clinical progression, but no increased risk for all-cause mortality. More importantly, these

findings were independent of traditional risk factors (i.e. age, sex, waist-to-hip ratio, and body mass index). A potential follow-up study could include asymptomatic participants (i.e. for primary prevention) or groups at increased risk for secondary events (i.e. coronary artery disease). Another study could assess whether changes in CAR across a given time period (i.e. increase or decrease in CAR) would relate to subsequent development of CV events.

Chapter 6 described the prognostic value of CAR for (CV) events and clinical progression, but CAR could not predict (CV) mortality in PAD patients. In 2016, we performed the CAR test in a group of healthy octogenarians who participated in the Nijmegen 4-Day Marches (n=85, aged 84±2). Using the Dutch National Death Registration, we obtained information on 75 participants (n=10 could not be found) and found that 8 participants have died. We found that those still alive had an average CAR of 2.6±1.6, whilst CAR was significantly higher in group that died across the 1year follow-up (5.8±2.7, $P<0.01$). The finding that CAR did not relate to all-cause mortality, is in line with our observations in **Chapter 6,** where also no relation was found for CAR *versus* all-cause mortality. Interestingly, although being underpowered, in **Chapter 6** we found that CAR was lower in those with CV-related mortality compared to the other PAD patients. Possibly, CAR relates to occurrence of CV-related mortality rather than all-cause mortality. Future studies are required to better understand this link, including our study in octogenarians.

Future perspectives for the CAR

The new CAR test provides a novel test that seems to reflect vascular health, which may fit with AHA-ACC criteria. First, the empirical background and physiological mechanism for CAR currently relies on literature on coronary responses to sympathetic stimulation. Studies in this thesis provide the first evidence for similarity between carotid and coronary artery responses to sympathetic stimulation. To further divulge the similarity between the carotid and coronaries and their physiological origin, future studies should be performed in a catheterisation laboratory, directly analysing endothelial function and sympathetic responses of both vessels. At least, our work provides early indication that the CAR represents a surrogate measure for coronary vascular health. Secondly, CAR is a feasible, short test (i.e. 4 minutes), supported by good reproducibility. Since many technical aspects of CAR are similar to FMD assessment, I recommend adherence to the expert-consensus guidelines for peripheral vascular assessment as described in this thesis when performing CAR tests. Additionally, the adopted methodology and technical aspects mentioned in this thesis can add to specific expert-consensus guidelines and a scoring tool for the CAR test. Finally, we have seen that CAR independently predicts cardiovascular events, but not all-cause mortality, in PAD patients, and is independent of structural measures. It is essential to confirm these findings in other (clinical) populations, to explore clinical and pre-clinical potency of CAR for evaluation of cardiovascular health. At least, data in this thesis warrant further exploration. Exploring carotid reactivity in response to ice may only be the tip of the iceberg.

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Chapter 8

Summary
Samenvatting
Portfolio
Dankwoord
List of publications
Curriculum Vitae

SUMMARY

Chapter 1 introduced the old and new measurement of endothelial function, and vascular health. The first part of this thesis focussed on the "old" flow-mediated dilation measurement, introduced in 1992, which relies on brachial artery vasodilation after a hyperaemic stimulus (e.g. after occlusion of a pneumatic cuff), and the susceptibility of the FMD measurement to variability.

In **Chapter 2** we sought for factors that might help to improve the reproducibility of the FMD measurement. We performed an analysis including 672 participants with repeated FMD. Overall we found an acceptable reproducibility, with 33% of the FMD measurements showing an excellent-to-moderate reproducibility. We identified several factors that independently increased the variation of the FMD, including the presence of hypertension, a lower resting FMD%, a larger baseline artery diameter, a longer time between subsequent measurements, and less laboratory experience with the measurement. Future studies should take these factors into consideration, as certain measures may lower variability of the FMD *or* more subjects should be included in individual studies when variation relates to non-modifiable factors.

Unfortunately, we also found that a large proportion of our study population demonstrated a moderate-to-poor reproducibility, despite all included studies adhered to expert-consensus guidelines. In **Chapter 3**, we described the relation between adherence to the expert-consensus guidelines and reproducibility of the FMD. In this meta-analysis, we combined data from twenty-seven studies, comprising 48 study groups, with a total of 1537 subjects. Adherence to expert guidelines was inversely related to the measurement error and adopting the guidelines (with specific notification of the use of a stereotactic probe-holder, continuous diameter recording and the use of automated wall-detection and analysis software) was crucial for improving the reproducibility of the FMD.

The second part of this thesis introduced the "new" carotid artery reactivity (CAR) measurement, which depends on carotid artery vasomotor responses following sympathetic stimulation (e.g. after a cold pressor test, CPT). The carotid artery appears to mirror coronary responses to CPT, as it shows dilation in healthy participants, whilst those at risk demonstrate a constriction. In this thesis, we sought to understand the relation of CAR with risk factors, and the similarity with the coronary responses. An attempt was made to unravel the underlying physiological mechanism of CAR. Finally, the prognostic value of CAR was examined in peripheral arterial disease patients, to further investigate to clinical potential of the CAR test.

In **Chapter 4** we explored the relation of CAR with cardiovascular risk, followed by assessing the similarity in response to sympathetic stimulation between the carotid artery and coronary arteries. We first compared CAR between 50 young and 44 older participants to assess relationships between CAR *and* traditional cardiovascular risk factors. We found

that CAR was lower in participants with ≥2 risk factors, compared to those with lesser risk factors. Secondly, we compared left anterior descending (LAD) artery velocity *with* carotid artery diameter in a subgroup of 33 participants, to assess similarity between coronary and carotid artery responses. We found that CAR correlated well with coronary artery velocity. This implies that CAR is related to increased CV risk and may represent a surrogate measure for coronary vascular health.

In **Chapter 5**, the physiological mechanism underlying the CAR was further explored, by examining carotid artery responses to different sympathetic stimuli (e.g. the cold pressor test [CPT] and the lower body negative pressure test [LBNP]), exploring the role of α_1-receptors, (nor) epinephrine receptors contributing to vasoconstriction, and assessing similarity between carotid and coronary arteries. First, 10 participants underwent both sympathetic tests in randomized order, whilst concurrently measuring CAR and coronary artery velocity. We found distinct carotid artery responses to different tests of sympathetic stimulation (e.g. dilation in response to CPT, and constriction following LBNP). Second, when measurements were repeated following α_1-receptor blockade by Prazosin, we found α1-receptors partly contributed to CPT-induced responses. Finally, we found agreement between carotid and coronary artery responses, during both types of sympathetic nerve stimulation as well as during α1-receptor blockade. These data indicate strong similarity between carotid and coronary responses to sympathetic tests and the role of α1-receptors.

Since the CAR test was newly introduced, the prognostic value of the test remained unknown and this question is highly relevant to understand its clinical utility. Therefore, in **Chapter 6**, we examined whether CAR predicts (cardiovascular) events in patients with peripheral arterial disease. A total of 172 PAD patients were included, and we recorded cardiac and cerebrovascular events, mortality and clinical progression to percutaneous transluminal angioplasty or loss of patency during a 12-months follow-up. We found that patients with carotid constriction showed a four-fold higher risk for cardiovascular events and two-fold increased risk for clinical deterioration, even after adjustment for other risk factors. This indicates that CAR provides a simple, novel strategy to predict CV events and progression in PAD patients, which has stronger prognostic value than current techniques.

Chapter 7 summarizes, discusses, and explains the findings of these studies, and aims to provide recommendations and implications. We discuss future prospects, and provide perspective for future vascular health assessment.

SAMENVATTING

Hoofdstuk 1 geeft een introductie in een oude en nieuwe meetmethode van de endotheel- en vaatfunctie. Het eerste gedeelte van dit proefschrift richt zich op de oudere methode, bloedstroom-gemedieerde vasodilatatie (FMD), welke afhankelijk is van een hyperemische prikkel (na afknelling) en in hoeverre deze meting ontvankelijk is voor variabiliteit.
In **hoofdstuk 2** hebben we de reproduceerbaarheid van deze FMD meting onderzocht. Voor deze studie zijn 672 proefpersonen met herhaalde metingen geïncludeerd. Ongeveer 33% van de FMD metingen resulteerden in een excellente-tot-gemiddelde reproduceerbaarheid. We hebben enkele factoren geïdentificeerd die onafhankelijk de variatie beïnvloeden, waaronder aanwezigheid van hypertensie, een lagere rust FMD, een grotere basis vaatdiameter, een langere periode tussen herhaalde metingen en minder ervaring met het uitvoeren van de FMD. Toekomstige studies zouden deze factoren in overweging moeten nemen om de variabiliteit van de FMD te verminderen.
Helaas vonden we ook dat een groot gedeelte van onze studiepopulatie een gemiddelde-tot-lage reproduceerbaarheid toonden, ondanks dat alle geïncludeerde studies de door experts-opgestelde richtlijnen volgden. In **hoofdstuk 3** onderzochten we de relatie tussen het volgen van de richtlijnen en de reproduceerbaarheid van de FMD. Hiervoor hebben we een meta-analyse uitgevoerd, met data van 27 studies, 48 groepen en 1537 proefpersonen. Wij vonden dat het volgen van de richtlijnen omgekeerd evenredig was met de meetfout. Het continu meten van de vaatdiameter, gebruik van automatische vaatwand detectie systemen en het gebruik van een probe-houder zijn essentieel voor het verbeteren van de reproduceerbaarheid van de FMD.

In het tweede gedeelte van dit proefschrift is een nieuwe meting geïntroduceerd, de carotis arteriële reactiviteit (CAR). Deze test meet de reactie van de carotis arterie (verwijding dan wel constrictie) na sympathische stimulatie (bijvoorbeeld de ijswater test, CPT, waarbij de linkerhand kort wordt ondergedompeld in ijswater om een sympathetische reactie uit te lokken). We hebben getracht het onderliggende fysiologische mechanisme te ontrafelen, welke terug te vinden zijn in de verschillende studies. Uiteindelijk hebben we de prognostische waarde van de CAR test bekeken in een patiëntengroep met perifeer vaatlijden, om de klinische waarde van de CAR verder te onderzoeken.
In **hoofdstuk 4** hebben wij onderzocht of de coronair arterie (de gouden standaard om de vaatgezondheid te bekijken) en de carotis arterie hetzelfde reageren na sympathische stimulatie. Bij 50 jongere en 44 oudere proefpersonen hebben wij de CAR gemeten. Vervolgens hebben we de associatie tussen CAR en traditionele risicofactoren bekeken. Ten slotte hebben wij de vergelijkbaarheid tussen de carotis en coronair arterie bekeken. We vonden dat de carotis meer verwijdt in jongere proefpersonen (hogere CAR), dat proefpersonen zonder risicofactoren een hogere CAR hebben dan mensen met 2 of meer

risicofactoren en dat de CAR goed relateert aan de coronair arteriën. Dit impliceert dat de CAR een waardevolle techniek is om het cardiovasculaire risico te evalueren.

In **hoofdstuk 5** hebben we het onderliggende fysiologische mechanisme verder verkend. Hiervoor hebben we de reactie van de carotis op andere sympathische stimuli (ijswater test en negatieve druk), de rol van α_1-receptoren (receptoren voor vasoconstrictie na een sympathische stimulatie) en de overeenkomst tussen de carotis en coronair arteriën bekeken. 10 proefpersonen hebben beide testen uitgevoerd, waarbij tegelijkertijd de carotis en coronair arteriën zijn gemeten. Deze metingen werden herhaald terwijl alle proefpersonen α_1-receptorblokkers hadden gebruikt. We vonden dat de carotis onderscheidend reageert op verschillende sympathische stimuli (dilatatie bij de ijswater test, constrictie bij de negatieve druk test), terwijl α_1-receptoren alleen een rol spelen bij de ijswater test. Tot slot vonden we sterke overeenstemming tussen de carotis en coronair arteriën, wat duidt op sterke vergelijkbaarheid tussen beide vaten in reactie op sympathische stimuli.

In **hoofdstuk 6** onderzochten we of de CAR test cardiovasculaire events (bijvoorbeeld een myocard infarct) kan voorspellen in patiënten met perifeer vaatlijden. In totaal zijn 172 patiënten geïncludeerd. Gedurende 12 maanden hebben we het optreden van cardio/cerebro vasculaire events, mortaliteit en klinische progressie gemonitord. We vonden dat patiënten met een CAR constrictie vier keer meer risico hadden op cardiovasculaire events en twee keer meer klinische achteruitgang, zelfs na correctie voor risicofactoren. Hieruit concluderen wij dat de CAR een simpele, nieuwe strategie is die cardiovasculaire events en ziekte progressie kan voorspellen in patiënten met perifeer vaatlijden.

Hoofdstuk 7 vat de resultaten van dit proefschrift samen, bediscussieert deze en tracht deze te verklaren. Ook geven wij aanbevelingen en implicaties van deze nieuwe test, waarmee wij ook een perspectief schetsen voor toekomstig vaatfunctie onderzoek.

Institute for Health Sciences
Radboudumc

PORTFOLIO

Name PhD candidate: ACCM van Mil	**PhD period:** *1-5-2014 – 1-7-2017*
Department: Physiology	***Promotor(s):*** *Prof. Thijssen, Prof. Hopman and Prof. Green*
Graduate School: Radboud Institute for Health Sciences	***Co-promotor(s):*** *Dr. Ellen Dawson*

		Year(s)	ECTS
	TRAINING ACTIVITIES		
a)	***Courses & Workshop***		
-	*Liverpool John Moores University Vascular sonography summerschool*	2014	1.5
-	*Radboudumc Introduction day*	2015	0.5
-	*Radboud Institute for Health Sciences Introduction course for PhD students*	2015	1.5
-	*Vascular Function and Angiogenesis in Health and Lifestyle related disease*	2015	5.0
-	*BROK (Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers)*	2015	1.4
-	*Biometrics course*	2015-2016	6.0
-	*Scientific Integrity*	2016	0.6
-	*PhD Organisation Nijmegen, course InDesign*	2016	0.5
-	*Management voor Promovendi*	2016	3.0
-	*Education in a Nutshell*	2016	3.0
b)	***Seminars & lectures***		
-	*Radboud Research Rounds*	2014-2017	0.5
-	*Prof. Paul Thompson (Lecture: Can [too much] exercise hurt your heart?)*	2015	0.1
-	*Prof. Duck-Chul Lee (Lecture: 'Health benefits of physical activity & fitness: What type of physical activity is best for health?')*	2017	0.1
-	*Prof. Bo Fernhall (Lecture: Inflammation, exercise and arterial function)*	2017	0.1
c)	***Symposia & congresses***		
-	*ARTERY Maastricht*	2014	0.5
-	*Radboud Research Round – vascular damage theme (Oral)*	2016	0.3
-	*Dutch Endothelial Biology Society, Biezenmortel (Oral)*	2016	1.3
-	*Okanagan Cardiovascular and Respiratory Symposium, Canada (Study collaboration)*	2016	1.3
-	*Radboud Into Frontiers (Poster)*	2016	1.0
-	*RIHS PhD retreat (Oral)*	2016	1.0
-	*European College of Sport Science, Essen (Oral)*	2017	1.3
d)	***Other***		
-	*Vascular Damage theme meetings*	2014-2017	0.5
	TEACHING ACTIVITIES		
e)	***Lecturing***		
-	*5O103: Beweging en sturing (practica)*	2014-2016	0.3
-	*HM03 - Clinical Exercise Physiology (Lectures, practica)*	2015-2017	1.5
-	*Minor10 – Practica echografie*	2016-2017	0.3
-	*1Mbe1 – beweging (werkgroepen)*	2016-2017	0.3
-	*LC circulatie-respiratie*	2016-2017	0.6
-	*Echo practica demo carrousel afdeling*	2014-2017	1.0
f)	***Supervision of internships / other***		
-	*Machteld van Erk (Master BMS) - Reproducibility of the carotid vasoreactivity test?*	2015	1.0
-	*Ralf Weijs (Bachelor BMW) - The effect of lifelong exercise participation on the protective effect of ischemic preconditioning on brachial artery ischemia-reperfusion injury in healthy older men*	2015	1.0
-	*Frederieke van Oorschot (Honours student Dick Thijssen) – daily supervision*	2015	0.3
-	*Nikki Bax (Master GNK Dick Thijssen) – daily supervision*	2015	0.3
-	*Jelmer Wilbrink (Master GNK Dick Thijssen) – daily supervision*	2016	0.3
-	*Thijs Kerstens (Honours student Dick Thijssen) – daily supervision*	2016	0.3
TOTAL			**38.2**

DANKWOORD

Nou, daar zit je dan, na 3 jaar ploeteren en direct doorgaan in een nieuwe functie, aan de eerste regels van je dankwoord. Het meest gelezen (en dus wellicht het belangrijkste?) stuk van het boekje. En een stuk gaat het worden, want wat hebben een hoop mensen met mij mee geleefd de afgelopen jaren. Samen met hen heb ik zware downs, maar gelukkig veel meer mooie ups meegemaakt. Want zo voelt promoveren wel een beetje, als een vrije val attractie, met grote hoogtes en diepe dalen. Ik had dit echt niet kunnen doen zonder de hulp en bijdrage van mijn collega's, vrienden en familie.

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CURRICULUM VITAE

Anke van Mil werd op 27 oktober 1989 geboren te ’s-Hertogenbosch. Na haar middelbare schoolperiode aan het Rodenborch College te Rosmalen, begon zij in 2008 met de opleiding Biomedische Wetenschappen, met als specialisatie Human Movement Sciences, aan de Radboud Universiteit te Nijmegen. Tevens heeft Anke de aanvullende master getiteld ‘Reflections on Science; the wider implications of cognitive neuroscience’ aan de Radboud Honours Academy gevolgd. Tijdens haar eerste onderzoeksstage kwam zij in aanraking met de afdeling Fysiologie. Onder begeleiding van Dr. Thijs Eijsvogels en Dr. Walter ter Woerds bekeek zij de effecten van warmte op de peri- en postoperatieve uitkomstmaten bij patiënten met totale heupvervangingen. In het afstudeerjaar van haar master heeft zij voor negen maanden in Cardiff, Wales gestudeerd en gewoond. Onder begeleiding van Dr. Eric Stöhr en Prof. Dick Thijssen heeft zij de effecten van warmte op hart- en vaatfunctie onderzocht. Deze stage heeft uiteindelijk de basis gelegd voor haar interesse in vaatfunctie metingen, waar haar verdere promotietraject zich grotendeels op heeft gericht. In mei 2014 is zij gestart met een onderzoeksproject in een samenwerkingsverband tussen de afdeling Fysiologie van het Radboudumc en de afdeling Humane Biologie van de Maastricht Universiteit. Dit project is verder uitgebreid tot een duaal promotietraject in een samenwerking tussen het Radboudumc en Liverpool John Moores University, het Verenigd Koninkrijk. Tijdens deze periode werden verschillende studies uitgevoerd naar de reproduceerbaarheid van de flow-gemedieerde vaatfunctie meting. Tevens is in deze periode de basis gelegd voor een nieuwe vaatfunctie test, de carotis arteriële vasoreactiviteit. De focus van het laatste jaar van haar promotie lag op de klinische relevantie en toepassing van deze nieuwe test. Tijdens haar periode als promovendus heeft zij meerdere bachelor- en masterstudenten begeleid en was zij betrokken bij het onderwijs van Geneeskunde en Biomedische Wetenschappen aan de Radboud Universiteit. Momenteel is Anke werkzaam op de afdeling Fysiologie als docent en studentcoördinator Fysiologie.

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