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Inter- and intra- raters reproducibility of flow-mediated slowing using local estimates of brachial artery pulse wave velocity

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palavras-chave desaceleração mediada pelo fluxo, velocidade da onda de pulso, reprodutibilidade, dilatação mediada por fluxo

resumo

INTRODUÇÃO: A desaceleração mediada pelo fluxo (DMF) mede de forma não invasiva a função endotelial braquial por meio de alterações na velocidade da onda de pulso (VOP) induzidas por uma hiperemia reativa, e é sugerido para mitigar as limitações bem conhecidas da dilatação mediada por fluxo (DMF), incluindo a reprodutibilidade subotima e a alta dependência da experiência do operador. No entanto, os poucos estudos que examinaram a reprodutibilidade da DMF mostraram resultados controversos, e usaram apenas medições regionais de VOP efetuadas por um único avaliador. Tal pode não refletir verdadeiramente as respostas exatas da rigidez da artéria braquial à hiperemia reativa e a sua aplicabilidade clínica. No presente trabalho avaliámos a reprodutibilidade inter- e intra-avaliadores das análises off-line do DMF através dos sinais brutos de distensibilidade arterial obtidos do procedimento DMF.
MÉTODOS: Vinte e quatro participantes saudáveis do sexo masculino com idades entre 23-75 anos, foram examinados por 2 avaliadores, em dois dias separados, para avaliar a reprodutibilidade inter- e intra-dias. As alterações na rigidez beta braquial e na VOP beta (DMF) induzidas pela hiperemia reativa foram calculadas de acordo com as fórmulas do fabricante através da utilização de um script R personalizado. A reprodutibilidade inter- e intra- avaliador foi examinada com coeficiente de correlação intraclasse (ICC), coeficiente de variação (CV) e gráficos Bland-Altman.
RESULTADOS: A reprodutibilidade inter- avaliador da DMS mostrou uma boa reprodutibilidade para as análises intra (bias: -0.12%; ICC: 0.84; 95% CI: 0.57 to 0.94; CV: 19%) e inter- dia (bias: -0.25%; ICC: 0.82; 95% CI: 0.57 to 0.92; CV: 16%). A reprodutibilidade intra avaliador mostrou uma reprodutibilidade moderada a boa para a análise inter- dia (1º Avaliador: bias: 0.39%; ICC: 0.78; 95% CI: 0.51 to 0.91; CV: 21%; 2º Avaliador: bias:0.14%; ICC: 0.53; 95% CI: -0.07 to 0.80; CV: 36%).
CONCLUSÕES: As análises off-line do DMS através dos sinais brutos de ultrassonografia são reprodutíveis entre diferentes avaliadores.

Keywords flow mediated slowing, pulse wave velocity, reproducibility, flow mediated dilation

abstract

INTRODUCTION: Flow-mediated slowing (FMS) measures brachial endothelial function non-invasively through reactive hyperemia-induced changes on pulse wave velocity (PWV), and it is suggested to mitigate well-known pitfalls of flow-mediated dilation (FMD) including suboptimal reproducibility and high-operator dependency. However, the few studies that examined FMS reproducibility have shown controversial results and used only regional measurements of PWV performed by a single rater This might not truly reflect the exact stiffness responses of the brachial artery to reactive hyperemia and limit its clinical usefulness. In the present study we assessed inter- and intra-raters reproducibility of off-line analyses of FMS using the raw distensibility signals obtained from the FMD procedure.
METHODS: Twenty-four healthy male participants aged 23 – 75 yr, were examined on two separate days to assess inter- and intra-day reproducibility. Reactive hyperemia-induced changes on brachial beta stiffness and beta PWV (FMS) were calculated according to the manufacturer's formulas using a tailored R-script. Inter- and int-rater reproducibility was examined with intraclass correlation coefficient (ICC), coefficient of variation (CV), and Bland-Altman plot estimates.
RESULTS: Inter-rater reproducibility of FMS showed an overall good reproducibility for both intra (bias: -0.12%; ICC: 0.84; 95% CI: 0.57 to 0.94; CV: 19%) and inter-day (bias: -0.25%; ICC: 0.82; 95% CI: 0.57 to 0.92; CV: 16%) analyses. Intra-rater reproducibility showed an overall moderate-to-good reproducibility for inter-day (1st rater: bias: 0.39%; ICC: 0.78; 95% CI: 0.51 to 0.91; CV: 21%; 2nd rater: bias:0.14%; ICC: 0.53; 95% CI: -0.07 to 0.80; CV: 36%) analyses.
CONCLUSIONS: Off-line analyses of FMS using raw signals from ultrasonography were reproducible among different raters.

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List of abbreviations

FMS Flow Mediated Slowing

PWV Pulse Wave Velocity

FMD Flow Mediated Dilation

PWVmin Minimum Pulse Wave Velocity

ICC Intraclass Correlation Coefficient

CV Coefficient of Variation

Dbas Baseline Artery Diameter

bPWV Brachial Pulse Wave Velocity

PWV_{bas} Pulse Wave Velocity Baseline

FMSabs flow-mediated slowing absolute

betaPWVbas baseline beta pulse wave velocity

bStiffness Overall: overall beta stiffness

NO Nitric Oxide

EDHF Endothelium-derived Hyperpolarizing Factor

ET-1 endothelin-1

VSM Vascular Smooth Muscle

eNOS Nitric Oxide Synthase

ACE Angiotensin-converting Enzyme

VEGF Vascular Endothelial Growth Factor

PDGF Platelet-derived Growth Factor

CVD Cardiovascular Disease

AUC Area Under the Curve

ED Endothelial Dysfunction

Dissertation Introduction

The endothelium is an inner layer of a vessel made up of epithelial cells present throughout the cardiovascular system (Hoskins et al., 2017).The endothelial function is responsible the exchange of fluids, regulation of vascular tone and by the formation of new blood vessels (Smith & Fernhall, 2011). In the past, the endothelium was considered a simple barrier, nowadays it is considered an organ where its proper functioning is crucial for good vascular health and that its dysfunction is an important factor in the development of vascular diseases (Smith & Fernhall, 2011). There are invasive and non-invasive ways to measure endothelial function, where in the Flow Mediated Dilation is the best-known non-invasive method to assess endothelial function (D S Celermajer et al., 1992). Despite being the most used worldwide in the context of research, it has some limitations such as the high cost of using ultrasound, the high dependence on the operator and poor reproducibility (Flammer et al., 2012; Dick H.J. Thijssen et al., 2019), which made its adoption difficult in a clinical context. The Flow Mediated Slowing (FMS) has emerged to overcome FMD limitations, for some authors as an alternative method (Telmo Pereira et al., 2018) and as a complementary method to others (Ellins et al., 2016). It has also been suggested that the FMS has better reproducibility compared to the FMD, but the existing literature is not only controversial but also used only regional measurements of PWV performed by a single rater and this might not truly reflect the exact stiffness responses of the brachial artery to reactive hyperemia and limit its clinical usefulness.

This dissertation is divided in 3 parts. Part 1 is a literature review where I start off with a first chapter addressing the entire vascular structure and its function, followed by a second chapter on evaluation methods to assess the endothelial function. Part 2 is a scientific manuscript that resulted from the development of this dissertation, and I present the closing remarks in Part 3.

Part 1

Literature Review

Chapter 1 – Vascular Structure and Function

Structure of blood vessels

Blood vessels are divided into two broad types of blood vessels, arteries, and veins (Hoskins et al., 2017). Arteries in both the pulmonary and systemic circulation carry blood away from the heart. These arteries have to deal with a relative high pressure, so they have a thick walls constituted by three layers (Hoskins et al., 2017). Looking from the inside out, we have the endothelium that is a semipermeable membrane layer made up of epithelial cells that covers all blood vessels of the cardiovascular system, making a biological interface between blood and tissues (Smith & Fernhall, 2011). The endothelium is positioned above the smooth muscle in the middle layer, its large surface facilitates its functions, including the exchange of gases and nutrients and the signaling of hemodynamics forces to underlying smooth muscle (Smith & Fernhall, 2011). The endothelium cells as well as cytoskeleton are linked to the basal lamina through integrins. The surface of the endothelium has the negatively charged layer called glycocalyx, which allows small water solutions to leave the circulation but functions to keep negatively charged proteins, such as lipoproteins and albumin in the bloodstream (Smith & Fernhall, 2011). Endothelial cells have a cytoskeleton, composed mainly of actin and myosin filaments, which support not only holding the endothelial cells together but also holding them together with the basal lamina, in addition, there are junctional proteins that bind endothelial cells to each other, they are dynamic structures that can change their functioning quickly in response to acute changes in permeability but their degree and functioning varies considerably throughout the vascular system (Smith & Fernhall, 2011). The next layer is the *tunica intima* which is the thinnest layer of the three layers having in its constitution endothelial cells, this one is in contact with the circulating blood. Arteries also have the *tunica media* which contains mostly smooth muscle cells and elastin fibers. Finally, we have the outermost layer, *tunica adventitia*, which is mainly made up of fibro-elastic connective tissue (Pugsley & Tabrizchi, 2000). Arteries can be divided into elastic arteries and muscular or distributive arteries, both of which have different properties in their wall (Hoskins et al., 2017). Elastic arteries, such as the Aorta, have a high ratio of elastin and collagen, which leads to greater distensibility to accommodate the high blood volume (Hoskins et al., 2017). On the other hand, the muscular or

distributive arteries are constituted by a thicker middle layer with less elastin and more smooth muscle, normally these are arteries that supply the organs (Hoskins et al., 2017). The veins bring the blood back to the heart opposite to arteries, having less pressure compared to the arteries and are consequently less thick. The elastin and collagen ratio is smaller, so the veins are more rigid than the arteries (Hoskins et al., 2017).

Endothelium - Function

In the beginning, the endothelium was considered a simple barrier, nowadays it is considered an organ whose functioning is crucial to maintain good vascular health and whose dysfunction is key in the initiation, progression and clinical complications of vascular disease (Cahill & Redmond, 2016). The endothelium has multiple functions in the blood vessels, his primarily function is regulate the exchange of fluids, nutrients, and gases between the blood and tissues, through glycocalyx and the intracellular junctions (Smith & Fernhall, 2011).

Below are described with more details two other major functions of the endothelium.

Vascular Tone Regulation

The endothelium has the function of regulating vascular tone, which is determined by the degree of smooth muscle contraction in the tunica media (Smith & Fernhall, 2011). The regulation of vascular tone, causing vasoconstriction or vasodilation, determine blood flow and ensure that organs receive a blood supply that matches the metabolic needs of the tissue, and has a big effect on blood pressure, and hence the force that blood exerts throughout the vascular system (Smith & Fernhall, 2011). Endothelial cells release chemical mediators like nitric oxide (NO), prostacyclin, PGI2, endothelium-derived hyperpolarizing factor (EDHF), endothelin-1 (ET-1), angiotensin II that leads to vasodilation and vasoconstriction, and the balance of these mediators determines blood flow distribution to various organs (Smith & Fernhall, 2011). The NO can cause vascular smooth muscle (VSM) relaxation, inhibits platelet aggregation, inhibits vascular smooth muscle proliferation, and inhibits the production of adhesion molecules (Smith & Fernhall, 2011), is the most important vasoactive substance, and its vasodilatory function is used as an index of endothelial function (Maiorana et al., 2003). Specifying in greater detail it’s vasodilator capacity, the ON is synthesized in the endothelium by the enzyme nitric oxide synthase (eNOS), which cleaves NO from the amino acid L-arginine, consequently, there is a rapid diffusion of ON from

the endothelium into vascular smooth muscle where it leads to relaxation (Smith & Fernhall, 2011). Both ON synthase and production is regulated by shear stress and by various receptor-bound agonists (Ando & Yamamoto, 2011; Smith & Fernhall, 2011). Shear stress is the force exerted on the vessel wall (endothelium) by the sliding action of blood flow, is the most important stimulus for the production of NO under normal conditions (Ando & Yamamoto, 2011; Smith & Fernhall, 2011). The endothelium identifies shear stress and cyclic strain as mechanical stimuli, which communicates to the interior of the cells, with this occurs an increase in the intracellular concentrations of Ca2þ and tetrahydrobiopterin (BH4)(cofactor of eNOS) and a activation of protein kinases which results in eNOS activation (Corson et al., 1996; Dimmeler et al., 1999; Fleming et al., 1998; Widder et al., 2007), this leads consequently to a change in cell morphology, function, and gene expression (Ando & Yamamoto, 2011). However, NO is not the only vasodilator released by the endothelium, when NO production is blocked through the infusion of an NO inhibitor such as LNMMA, vasodilation is greatly diminished but not completely abolished, suggesting that other vasodilators are also involved (Smith & Fernhall, 2011). Oppositely, the production of the vasoconstrictors like ET-1 and cell surface expression of angiotensin-converting enzyme (ACE), which generates the potent vasoconstrictor angiotensin II, decreases in response to laminar shear stress (Rieder et al., 1997; Sharefkin et al., 1991). The endothelium controls this relationship between vasodilation and vasoconstriction but the actual vasomotion is produced by smooth muscle contraction or relaxation, through several processes initiated by the endothelium (Koller et al., 1994; Williams & Lind, 1979). Important to note that these vasoactive agents produced in the endothelium are secreted as they are produced, meaning that they are not stored for later release (Herring & Paterson, 2018). An artery can respond to minute-to-minute changes in hemodynamics, the vessels must adapt to differing physiologic demands and conditions from changes in blood pressure and flow (Wootton & Ku, 1999). In a long term, an artery can adapt to increases or decreases in wall shear stress, with the increasing of wall shear stress, the expected response is vasodilation of the artery and in turn the remodeling to a larger diameter with the same arterial structure (Wootton & Ku, 1999). On a timescale of weeks to months, arteries will remodel their intima and media layers, the medial thickness is influenced by the local amount of hoop stress (stress along the tangential direction of the arterial wall's cross section) and nutrition (Wootton & Ku, 1999) As the blood pressure increases, the hoop stress will proportionally increase (Clark & Glagov, 1985), due to the formation of a lamellar unit requires the proliferation of smooth muscle

cells and the creation of a highly organized extracellular structure, the process may take several days (Wootton & Ku, 1999). Alterations in the pulsatile pressure lead to changes in organization of the elastin and collagen structure within the media (Glagov et al., 1988; Rodbard, 1970).

Angiogenesis

The endothelium participate in the development of new vessel formation, denominated by Angiogenesis (Smith & Fernhall, 2011). There are four important factors that increase growth of new blood vessels have been found being them the vascular endothelial growth factor (VEGF), fibroblast growth factor, platelet-derived growth factor (PDGF), and angiogenin, presumably, the deficiency of tissue oxygen or other nutrients, or both, that leads to formation of the vascular growth factors (also called "angiogenic factors") (Hall, 2016). The endothelial cells can be stimulated to split rapidly when there is a need for new vessel formation. This process begins with the breakdown of the basal lamina and the sprouting of the endothelium from the side of a capillary or venule. The cell extensions put out by the endothelium (pseudopodia) grow toward the stimulus for new blood supply. These pseudopodia are enlarged by cytoplasmic growth until they divide into daughter cells. Vacuoles then consume material within the new descendant cells. Eventually the vacuoles of the daughter cells fuse, resulting in a new lumen. The entire process continues until the new sprout encounters another capillary to connect to (Smith & Fernhall, 2011).

Endothelial - Dysfunction

Endothelial dysfunction is a broad term that typically defines endothelial pathology characterized by an amplified permeability to plasma lipoproteins, increased adhesiveness to leukocytes, and disparities in the release of factors that regulate vascular tone and hemostasis (Smith & Fernhall, 2011). The NO bioavailability is reduced, there is an imbalance between vascular endothelial growth factor and NO, and impairment of endothelial repair (Shi & Vanhoutte, 2017). Such dysfunction shows a tendency to vasoconstriction, pro-thrombotic, and pro-inflammatory states (Avogaro et al., 2011; Widlansky et al., 2003). The endothelial dysfunction manifests itself in the early stages pathogenesis of atherosclerosis and is associated with increased incidence of cardiac events (D S Celermajer et al., 1994; Neunteufl et al., 2000).

Endothelial dysfunction is usually caused by injury or death of endothelial cells, in the most extreme case, a significant injury leads to endothelial cells desquamation from the vessel lining. Healthy endothelial cells effectively cover a collagen-containing basement membrane preventing its interaction with circulating thrombocytes (Pober et al., 2009). This desquamation exposes this platelet-activating surface and results in thrombosis, i.e., a failure to keep blood in a fluid state (13). Apoptosis is defined has a programmed cell death mediated by the activation of intracellular proteases called caspases, in the most cases of endothelial cells death appear apoptotic (Pober et al., 2009), this term was first used to describe a pattern of programmed cell death observed in the maturing embryo (Kerr et al., 1972; Majno & Joris, 1995). There are several harmful stimuli that can trigger the death of endothelial cells by apoptosis, environmental stress is one of them, which can be divided into oxidative stress, endoplasmic reticulum stress, metabolic stress and genotoxic stress (Pober et al., 2009). There are other stimuli that can cause endothelial cell death including those used by innate and adaptive immunity such as signaling by death receptors and by other immune (Pober et al., 2009).

Chapter 2 - How to evaluate Endothelial Function and the Mechanical Properties of the Arteries

It is important to evaluate the function of endothelium due to its association with cardiovascular risk factors and diseases (Moens et al., 2005; Münzel et al., 2008), and early onset in the process of atherosclerosis (Münzel et al., 2008). There are various techniques for assessing endothelial function, they can be either invasive or noninvasive, and assess different aspects of pathobiology (Al-Qaisi et al., 2008). For the assessment of preclinical disease, a noninvasive, reliable, reproducible, cheap, and easy to perform technique is suggested (Deanfield et al., 2007).

Invasive methods

Vasoactive agents are delivered via intra-arterial infusion, whilst the response is measured with high resolution ultrasound or strain gauge plethysmography. In addition, intravascular infusions of vasoactive stimulants can be combined with intravascular ultrasound. For example, intravascular coronary flow-mediated dilatation (FMD) studies (Halcox et al., 2002) or studies of radial artery grafts used for coronary bypass surgery (Chong et al., 2006).

Flow Mediated Dilation - Non-invasive method

Noninvasive methods of measuring endothelial function include ultrasound FMD, salbutamol-mediated endothelial function measured by pulse wave analysis or pulse contour analysis , flow-mediated magnetic resonance imaging, laser Doppler flowmetry, and flow-mediated pulse amplitude tonometry (Al-Qaisi et al., 2008). The FMD technique was first introduced by Celermajer et al., (1992), and represents a popular and widely non-invasive method for examining brachial artery endothelium-dependent dilation. The FMD standardized procedure aims to create an ischemia period in the forearm by inflating a forearm cuff above systolic blood pressure for 5 min. After this ischemia period, the cuff is rapidly deflated, and blood flows into the distal portion of the arm – reactive hyperemia, with the changes in brachial artery diameter and blood flow velocity being quantified using ultrasonography. During reactive hyperemia, the increase in blood flow causes an increase in shear stress on the endothelium, which in turn releases

NO and causes arterial dilation. The FMD procedure is normally expressed as a percent change in vessel diameter from baseline to peak diameter observed following reactive hyperemia, a greater percent change is generally assumed to be indicative of a healthy endothelium, whereas an abolished or reduced flow-mediated dilation may be indicative of endothelial dysfunction (Flammer et al., 2012; Smith & Fernhall, 2011). Impaired FMD has been demonstrated to be an early step in the development of subclinical target organ damage and late clinical events (Charakida et al., 2010). Brachial FMD is associated with carotid intima-media thickness progression in a population free of cardiovascular disease (CVD) and in hypertensive postmenopausal women (Rossi et al., 2011). Some studies report a significant 8–13% lower risk of cardiovascular events per percent point increase in brachial artery FMD (e.g. from 5% to 6% dilation), both in high- and low-risk populations (Inaba et al., 2010; Xu et al., 2014), although it is more pronounced in patients with established CVD (Matsuzawa et al., 2015; Ras et al., 2013).

Flow Mediated Dilation - Protocol

FMD technique adopts ultrasound to examine changes in brachial artery diameter. Its application brings several technical considerations in terms of subject preparation, image acquisition and site selection, sphygmomanometer probe position, cuff occlusion time, the accurate use of edge-detection software as well as the correct characterization of the FMD response (Flammer et al., 2012).

Subject preparation

Protocol pre-requisites are a very important part of the protocol. Numerous studies fail on the control these factors and/or the report on their procedures in sufficient detail (Dick H.J. Thijssen et al., 2019). Participant-related factors, such as food ingestion, alcohol consumption, smoking, supplements, drugs, physical activity, and mental stress, have all be shown to influence FMD (Dawson et al., 2013; Hijmering et al., 2002; Papamichael et al., 2005; Dick H J Thijssen et al., 2011). In a way to diminish their effect, it is recommended that subjects should be fasted (>12h), avoid exercise (>24 h), and refrain from caffeine, vitamin C, polyphenols, alcohol, and supplements known to affect the cardiovascular system for a consistent period (typically >12 h),

previously to FMD evaluation. Smokers must refrain from smoking for a standardized period (preferably >6 h) (Dick H.J. Thijssen et al., 2019).

Cuff position

The occlusion cuff position is important because it can cause a change in magnitude (Naylor et al., 2005), duration (Doshi et al., 2001; Naylor et al., 2005) nature (Green et al., 2014), and possibly the clinical relevance (Green et al., 2011) of the dilator response. The dilation in response to distal cuff occlusion is about 70% NO-mediated, whereas proximal cuff placement is only about 30% NO-mediated (Green et al., 2014). This is an important mechanistic aspect because some studies specifically aim to study the NO-dependent pathway, given its established importance in the process of atherosclerosis. Thus, strict standardization of distal cuff occlusion (i.e. below the imaged artery) is recommended to ensure maximal dependence of the dilator response on endothelium-derived NO (Dick H.J. Thijssen et al., 2019).

Duration and magnitude of cuff occlusion

Cuff occlusion duration impacts the dilation of the artery, a short period of cuff occlusion (<5min) leads to a conditioned dilation (Leeson et al., 1997), whereas longer periods lead to larger blood flow and diameter responses but maybe less tolerable for volunteers (Naylor et al., 2005). Thus, given the nature of dilation changes with longer periods of occlusion (Mullen et al., 2001), it is recommended to use a 5-min cuff occlusion protocol. Most studies use a pre-defined cuff occlusion pressure (typically between 200 and 300mmHg) but is important that cuff pressure should exceed >50mmHg above systolic pressure to prevent arterial inflow (Dick H.J. Thijssen et al., 2019). A reasonable recommendation to minimize subject discomfort is to use a smaller-sized (5 cm), pediatric cuff instead of a standard (12 cm) cuff. In addition, providing clear information about the study protocol and FMD-procedure is essential (Dick H.J. Thijssen et al., 2019).

Baseline and Post-deflation Measurements

The results of the FMD can be expressed as the absolute delta score (Δ) and percentage change between post-deflation peak diameter and brachial artery baseline diameter as follows: i

$FMD\ (mm) = peak\ diameter_{post-ischemia} - D_{bas}$ and ii $FMD\ (\%) = \frac{absolute\ change}{D_{bas}} \times 100\%$, respectively (Harris et al., 2010; Dick H.J. Thijssen et al., 2011, 2019). The FMD is a function of the degree of reaction to stimuli, but also of baseline vasomotor tone and structural remodeling (Dick H.J. Thijssen et al., 2019). Most studies and FMD guidelines use the pre-inflation diameter to calculate the FMD. It is recommended that the baseline diameter is recorded for at least 30 seconds (Dick H J Thijssen et al., 2011). As the FMD is defined as an increase in diameter after occlusion, baseline diameter is generally inversely and moderately correlated with FMD (Atkinson & Batterham, 2013a; D S Celermajer et al., 1992; Silber et al., 2005). This association is of utmost importance when comparing groups that have different baseline diameters (age and CVD) (van den Munckhof et al., 2012; Yeboah et al., 2007) and for interpretation exercise intervention studies that may affect resting diameter (Green et al., 2017).

In terms of post-deflation measurements, peak diameter was initially propose to be recorded for 60 seconds after ischemia, but this can significantly underestimate the true peak dilation, and the time to peak dilation may differ between groups (Black et al., 2008) or after interventions (Black et al., 2008; Liuni et al., 2010; Padilla et al., 2009; D H J Thijssen et al., 2011). Thus, to guarantee a successful evaluation of the true peak diameter of the brachial artery, guidelines support continuous examination up to 180-s post- deflation (Dick H.J. Thijssen et al., 2019). Important to note that the nature of the diameter responses can be different between arteries, the content of endothelial NO synthase is heterogeneous throughout the arterial tree (Dick H.J. Thijssen et al., 2008), in case of lower limbs for instance a period of time above 3 minutes must be considered, these potential differences must be considered when performing and interpreting the FMD depending the arteries (Dick H.J. Thijssen et al., 2019).

There are temporally distinct FMD patterns to maximal dilation. There are the early dilators, subjects who have a maximal dilation at 50-s after ischemia, the late dilators who have a maximal dilation over 50-s and the non-responders, those who do not dilate at all (Irace et al., 2016). Additionally, the nature of the diameter responses can be different between arteries, the content of endothelial NO synthase is heterogeneous throughout the arterial tree (Dick H.J. Thijssen et al., 2008). More so, Irace et al., (2014) suggested that not only the magnitude of the FMD is important in identifying patients at risk of cardiovascular disease, but also the latency. At

a similar magnitude of FMD, delayed vasodilation is associated with a higher CVD risk score. Low CVD risk subjects show a marked and early FMD response after ischemia.

Blood Velocity and Shear Rate

Duplex ultrasound-derived blood velocity and diameter data are both required for calculating shear stress response post-deflation, the eliciting stimulus for artery dilation during FMD. The blood velocity, baseline, have a crucial role in the calculation of the area under the curve (AUC) method to assessing shear rate (Pyke & Tschakovsky, 2007). A precise assessment of resting blood velocity is essential, blood velocity can vary substantially over time, consequently, is recommended that baseline blood velocity be averaged for a minimum of 10 to 20 seconds (Gill, 1985). A clear description of how the blood velocity was measured must be mentioned. Mean velocity can be calculated by taking half of the peak velocity (i.e. fastest-moving blood cells in the center of the vessel), or by the intensity weighted mean velocity (i.e. mean velocity from all Doppler shifts across the vessel) (Thrush & Hartshorne, 2010).

The increase in shear stress is the physiological stimulus for dilation, a large part of the studies present shear rate, assuming that blood viscosity is kept constant, and that does not differ between participants and/or groups (Boot et al., 2002; Gnasso et al., 1996; Padilla et al., 2008). There are two different formulas, according to the sample volume size and placement (Parker et al., 2009): a. Shear rate = 8 * mean blood velocity/internal diameter; for large, centered sample volume; b. Shear rate = 4 * mean blood velocity/internal diameter; for small, centered sample volume. There is no uniformity in the calculation of shear rate calculations. Therefore, it must be described in each manuscript (Parker et al., 2009).

FMD Normalization

Different studies have used various approaches to shear stress stimulus, based on the importance of shear stress as the provoking stimulus and the assumption that the response variation of FMD within and between subjects are related to the magnitude of reactive hyperemia (Dick H.J. Thijssen et al., 2019) An early approach involved a ‘simple’ ratio normalization by dividing FMD by shear rate stimulus (De Groot et al., 2004; Padilla et al., 2008; Parker et al., 2006). Yet, this approach inclines to the violation of important statistical assumptions: (a) the relationship between

both parameters is linear, (b) the intercept for the regression slope of this relationship is zero, (c) data (including residuals) are normally distributed, (d) variances are similar between groups, and (e) the ratio does not lead to spurious correlations with other variables (Atkinson et al., 2009). Whilst acceptable-to-good relation between the shear rate stimulus and FMD is present within-subjects (Betik et al., 2004; Leeson et al., 1997; Padilla et al., 2008; Pyke et al., 2004; Pyke & Tschakovsky, 2005, 2007), studies examining this relation between subjects was found to be weak (Dick H.J. Thijssen et al., 2008) or absent (Dick H.J. Thijssen et al., 2009). Whilst this does not invalidate the role of shear stress as the dilator stimulus, it indicates that FMD variability cannot be simply controlled for by ratio normalization (Atkinson et al., 2009).

Other studies have taken a statistically approach to normalize FMD-responses to the shear stress stimulus, including shear rate as a covariate in an analysis of covariance (Atkinson et al., 2009; Atkinson & Batterham, 2013b; Harris & Padilla, 2007). Covariate FMD to shear rate is, nevertheless, misleading as the covariate (i.e. shear rate) is associated with the independent and/or outcome variable (e.g. age, sex, baseline diameter, and FMD). Yet, since calculations of shear rate mainly depends on baseline diameter, shear rate normalization may not be necessary if the FMD is scaled correctly to baseline diameter applying an allometric scaling approach (Atkinson, 2014). Currently, it is not defined which statistical approach is best to normalize FMD responses (Dick H.J. Thijssen et al., 2019)

Vessel diameter assessment - automated vs non-automated software

The initial FMD studies used a manual strategy to assess diameter change through visual inspection and calipers placement (D. S. Celermajer et al., 1992; David S. Celermajer et al., 1994). This method is highly operator-dependent, carries the risk of observer error, and is time-consuming (Harris et al., 2010; Mancini et al., 2002; Preik et al., 2000; Williamson et al., 2008; Woodman et al., 2001). Software systems for automatic diameter measurement have been developed over the years, promoting time-efficient approaches and limited operator-related errors and bias. Intra-observer variation is significantly lower with automated analysis compared to the classic manual technique (Gemignani et al., 2007; Mancini et al., 2002; Preik et al., 2000; Sonka et al., 2002; Williamson et al., 2008; Woodman et al., 2001). Maximal brachial vasodilation is 0.1 to 0.4 mm and the common resolving power of manual calipers is 0.1mm. Thus, the manual approach is likely

to introduce significant with- in- and between-observer error (Dick H.J. Thijssen et al., 2019). Manual measurements have a low number of samples per timeframe as well, at the same time as a more detailed time-course of changes in diameter is advantageous, particularly to identify the true peak diameter (Black et al., 2008). Within the advantages of automatic systems, real-time analysis allows for instantaneous feedback on scan quality and rapid adjustments in response to patient/probe movements. This may help the technician to optimize image quality. Another advantage is to immediately identify the technical failure of the measurement, with the possibility to repeat the FMD after an adequate resting time (Dick H.J. Thijssen et al., 2019). Another advantage is the ability to use both ECG-gated and non-gated ultrasound images without affecting the FMD results (Gemignani et al., 2008). This can keep the experimental structure as simple as possible by adopting a cheaper entry-level ultrasound device without ECG synchronization capability. The automatic FMD evaluation method must be practical, easy to use, and should provide options as well to track different parameters (i.e. shear rate). Moreover, the high amount of data, ease of use, and other technical features may help adequate training of the operator. Lastly, any new automatic system must be validated in appropriate populations before starting to be used in laboratories (Faita et al., 2011; Woodman et al., 2001).

Flow Mediated Dilation - Limitations

The FMD has its limitations. Firstly, this technique uses ultrasound equipment as its main tool, which makes it a relatively expensive technique (Dick H.J. Thijssen et al., 2019). Secondly, the technical application of this method is challenging and requires an extensive period of training and standardization (Flammer et al., 2012). This leads to another limitation, namely, its poor reproducibility. Extensive periods of training and technique standardization decrease the inter- and intra-user variability, but are time-consuming (Dobbie et al., 2020; Ellins et al., 2016; Flammer et al., 2012; Dick H.J. Thijssen et al., 2019). Dobbie et al., (2020) analyzed the validation of semi-automated FMD measurement in healthy volunteers and showed an intraclass correlation coefficient (ICC) of 0.334 and a coefficient of variation (CV) of 45.87% for automated analysis, and an ICC of 0.815 and a CV of 11.40% for manual analysis, concluding that automated analysis

had poor reproducibility and manual analysis had good reproducibility. Another paper from Harris et al., (2007) showed a mean ICC from FMD of 0.911 and an average CV of 25%, thus concluding that the FMD is reproducible according to the authors. Another group of investigators (Ghiadoni et al., 2012) showed CV's of 9.9±8.4 and 12.9±11.6% for the intra-session and inter-session FMD measures, respectively. This suggests that FMD assessment is highly reproducible in a group of healthy volunteers. Still, the CV's reported are often very large, even when adhering to standardized guidelines (CV > 10%) (Bots et al., 2005; Stoner & Sabatier, 2012).

Flow-mediated slowing - An alternative method

FMS is a Pulse wave velocity (PWV)-based index, which is the speed of the pulse wave generated by the heart, along the arterial tree (Safar et al., 2015). It represents the simplest way to measure the stiffness of a specific arterial segment, as it is noninvasive, reproducible, and supported by considerable scientific literature (Salvi, 2017). The PWV is inversely related to the viscoelastic properties of the wall itself, so the higher the velocity, the less elastic the wall (Salvi, 2017). This is particularly important as arterial stiffness is considered an important factor of the development of cardiovascular complications and has gained recognition as a risk factor in patients with arterial hypertension and other CVD (Mancia et al., 2013; Milan et al., 2011, 2013). The PWV is not equal along the vascular tree, in central arteries like aorta, the PWV is much slower indicating a more compliant or less stiff artery, than the in a peripheral artery like brachial artery (Smith & Fernhall, 2011). This differences in PWV across the vascular tree are likely due to the difference in arterial wall composition, for example, the elastic central arteries have less smooth muscle but more elastin, while peripheral arteries have a substantial amount of smooth muscle and collagen but less elastin (Smith & Fernhall, 2011).

FMS is defined as the minimum pulse wave velocity (PWVmin) during reactive hyperemia (Cauwenberghs et al., 2018; Ellins et al., 2016; Naka et al., 2006; Telmo Pereira et al., 2018; Rusak et al., 2010). According to the Moens–Korteweg equation $PWV = \sqrt{\frac{E \cdot h}{d \cdot \rho}}$, PWV is directly proportional to the arterial wall width and Young's modulus, and inversely proportional to blood viscosity and vessel diameter (Caro et al., 2011), therefore, when the vessel diameter increases during FMD, due to endothelium-dependent vasodilation (Stoner et al., 2012; Stoner & Sabatier,

2012), the endothelium-dependent increase in diameter would be expected to result in a reciprocal decline in PWV (Stoner et al., 2020).

The FMS emerged to overcome the limitations of the FMD, reproducibility being one of them, as mentioned above. Ellins et al., (2016) compared the reproducibility of the FMS, using an oscillometric technique, with the reproducibility of the FMD, and found FMS to be reproducible (CV=7%) compared to FMD (CV=27%). On the other hand, Marôco et al., (2021)when comparing the reproducibility of FMS through applanation tonometry method with FMD, showed that FMS is not a reproducible technique, obtaining a CV above 100%.

Dissertation Purpose

The purpose of this dissertation can be divided into three main points, one of which being to understand the relevance of using FMS in vascular function evaluation. Another point is to analyze the FMS reliability through an offline analysis of the raw FMD signal from a single or multiple raters. The last point is to know what new conclusions we were able to reach and what future directions are related to the assessment of vascular function.

Part 2

Introduction

Endothelial dysfunction (ED) is characterized by a reduction in nitric oxide bioavailability, an increase in permeability to plasma lipoproteins as well as adhesiveness to leukocytes (Smith & Fernhall, 2011). The assessment of ED is of clinical relevance as it is the early event that precedes clinical manifestations related to atherosclerosis, and has been shown to independently predict all-cause and cardiovascular mortality (Matsuzawa et al., 2015; Xu et al., 2014)

Brachial artery flow-mediated dilation (FMD) is the most used and accepted non-invasive method to quantify endothelial function (D. S. Celermajer et al., 1992; Flammer et al., 2012). Despite its acceptance, FMD is highly operator demanding and presents a suboptimal reproducibility even when adhering to standardized guidelines (CV > 10%) (Bots et al., 2005; Stoner & Sabatier, 2012), which precludes its use in clinical settings (Ellins et al., 2016; Naka et al., 2006; Stoner et al., 2020). To overcome these well-known pitfalls of FMD, the flow-mediated slowing (FMS) has emerged as a methodological alternative (Ellins et al., 2016) to indirectly access endothelial function through reactive hyperemia induced changes on segmental pulse wave velocity (PWV) (Naka et al., 2006) – a surrogate marker of arterial stiffness (Kucharska-Newton et al., 2019; R. et al., 2015). From a mechanistic perspective, the reactive hyperemia should reduce PWV under healthy conditions through the NO-mediated vasodilation, as vessel diameters are inversely proportional to PWV as stated in the Moens–Korteweg equation (Hoskins et al., 2017).

Seminal –research showed that segmental FMS had superior reproducibility (CV: 7%) than the FMD procedure (CV:27%)(Ellins et al., 2016). However, not only this was a single rater study, ruling out assumptions on its clinical relevance wherein multiple raters would evaluate the same patient, as these lower CVs have not been able to be replicated as reported by a recent study (Marôco et al., 2021) who reported higher inter-day coefficients of variation for FMS (CV: 119%) when compared to FMD (CV: 25%). One possible reason for this discrepancy is the heterogeneity in viscoelasticity properties between the upper limb arterial segments used to estimate PWV. For example, Ellins et al., (2016) estimated FMS using reactive hyperemia induced changes in PWV over the brachial to radial arterial segment, which is a muscular arterial segment with thicker tunica media. On the other hand, Marôco et al., (2021) used the PWV of the carotid-radial segment which has higher elastin to collagen ratio resulting in a greater distensibility.

From a methodological standpoint, it is plausible that the use of segmental PWV measurements does not reflect the exact stiffness responses of the brachial artery to reactive hyperemia, given the poor agreement between local (e.g. carotid PWV) and segmental measures of PWV (carotid-femoral PWV)(Lim et al., 2016). In addition, the distance measurement between arterial sites of interest required to estimate segmental PWV is the main source of inaccuracy (R. et al., 2015; Segers et al., 2009) as the measurement of travel distances on the surface of the body may not accurately represent the true length and anatomy of the arterial segments, especially in people with obesity, and when arteries become increasingly tortuous with age (Sugawara et al., 2008). To circumvent these issues, mathematical models derived from the Bramwell & Hill, (1922) equation have been developed to estimate PWV using a single arterial site (Weber et al., 2015) or local PWV of a single vascular bed (Tânia Pereira et al., 2015). Thus, ultrasound-based methods are increasingly used to access the local mechanical properties of arterial walls from changes in pressure that dictate volume fluctuations, without the need for a circulatory model (Laurent et al., 2006). Importantly, local PWV derived from ultrasound pressure and diameter measurements conducted simultaneously with FMD is likely more in measuring local artery stiffness responses to reactive hyperemia (Tânia Pereira et al., 2015), and thus could help clarify the controversial reproducibility and relevance of FMS as a complement (Ellins et al., 2016) to the FMD procedure.

Therefore, the purpose of this study was to examine the reproducibility of single and multiple raters at measuring local FMS using ultrasound.

Methodology

Participants

Twenty-four male participants, healthy and physically active, aged 23 – 75 yr, were recruited for this study. Smoking, cardiovascular (e.g., heart failure, coronaropathy), metabolic (e.g., diabetes mellitus), and renal diseases were the exclusion criteria. All participants reported to the laboratory on a fasted state (≥ 6h) and refraining from strenuous exercise, vitamin supplements, foods/drinks containing caffeine and alcohol ≥ 12h (Dick H.J. Thijssen et al., 2019). Inter-day reproducibility was assessed on two occasions always at the same time of the day (in the morning) with a minimum of 48h between sessions. Intra-day reproducibility was assessed with two measurements performed 20 min apart. All participants gave written informed consent after a detailed explanation of the experimental procedures and aims of the study. All experimental procedures were approved by the ethics committee of Faculdade de Motricidade Humana – Universidade de Lisboa (10/2020) and were aligned with the Declaration of Helsinki for human research.

Flow-mediated dilation

FMD was assessed in the right brachial artery with an ultrasound equipped with a 7.5-MHz linear array probe incorporating a 5-MHz Doppler transducer, placed ~4 cm above the antecubital fossa, and held by a mechanical clamp (Arietta V60, Hitachi Aloka Medical Ltd, Mitaka-shi, Tokyo, Japan) following standard guidelines (Corretti et al., 2002; Dick H.J. Thijssen et al., 2019). Before each measure, participants were in a supine position for 15 min with their right arms extended <80° laterally from the torso and at the level of the heart, in a quiet climate control room (22-24º C). Reactive hyperemia was induced by rapid cuff-deflation following a forearm occlusion maintained for 5 min at 250 mmHg. Brachial artery diameter was measured with automated edge-detection software allowing precise measurement of the artery diameter (Dick H.J. Thijssen et al., 2019). Brachial baseline artery diameter (Dbas) was averaged in end-diastole during the last 60-s of the baseline period, whereas the highest 10-s average interval throughout the first 3- minute collection period after cuff-deflation represented peak hyperemic diameter. FMD was calculated as $FMD\ (mm) = peak\ diameter_{post-ischemia} - D_{bas}$ and a the ratio $FMD\ (\%) = \frac{absolute\ change}{D_{bas}} \times 100\%$. Doppler measurements of peak hyperemic blood velocity were performed at an isonation

angle of ≤ 60º (Dick H.J. Thijssen et al., 2019). All image acquisitions and analyses were performed by the same researcher who had more than 100 hours of experience.

Flow-mediated slowing

Brachial PWV (bPWV) and consequently FMS were estimated from distensibility and blood pressure raw signals of the FMD procedure using the manufacturer equations (eq.1 and eq.2). Systolic and diastolic blood pressures required to estimate beta stiffness and bPWV were recorded using finger plethysmography on a beat-by-beat basis (Finapres® Nova, Ohmeda, Louisville, Colorado, USA), and were then averaged (95% trimmed mean) over the duration of the FMD procedure. Systolic and diastolic blood pressure were Baseline bPWV was averaged during end-diastole during the last 60-s of baseline, whereas min $bPWV_{ultra}$ was determined from time bins of 5-s during the first 3 minutes after cuff-deflation. FMS was calculated as the absolute-change $FMS\ (m.s^{-1}) = bPWV_{post-ischemia} - bPWV_{baseline}$ and percentage change as follows $FMS\ (\%) = \frac{absolute\ change}{PWV_{\ baseline}} \times 100\%$ (Ellins et al., 2016; Telmo Pereira et al., 2018). All of these analyses were conducted offline using R software using a tailed R script.

$$(eq.1)\ \beta = \ln\left[\frac{SBP/DBP}{(D_{syst} - D_{diast})/D_{diast}}\right]$$

Where, b is beta stiffness; SBP, brachial systolic blood pressure, DBP, diastolic blood pressure; D_{syst}, brachial artery diameter during systole; D_{diast}, brachial artery diameter during diastole.

$$(eq,2)\ bPWV = \sqrt{((\beta \times DBP)/(2\rho)}$$

Where, bPWV is brachial artery pulse wave velocity; b, beta stiffness, DBP, brachial diastolic blood pressure; and r, is blood density - assumed constant (1050 kg/m^3).

Statistics analysis

Based upon an ICC estimate of 0.80 from the FMD reproducibility results of high specialized vascular laboratories (operators with complete certification process where 10 repeat scans with a coefficient of variation <2% %FMD are required) (Charakida et al., 2013), an à priori power analysis using R package ICC.Sample.Size (Rathbone et al., 2015) suggested that 24 participants were necessary to ensure good reproducibility in intra and inter-day repeated measurements (α= 0.05, 1−b =0.90, k =2, null hypothesis = 0.40). The distributions of bPWV, and FMS were tested for normality with the Shapiro-Wilk test and plot representation. Reproducibility assessment of FMS analyses within and between raters was conducted using the coefficient of variation (SD/Mean * 100) and two-way absolute agreement mixed models intraclass correlation coefficient (ICC (2,1)) computed with irr package. The ICC was interpreted as following: poor < 0.50, moderate [0.50, 0.74], good [0.75, 0.90], and excellent >0.90 (Koo & Li, 2016) Bland-Altman plots were also used to evaluate the reproducibility of FMS_{ultra}.using ggplot 2 package. All statistical analyses were conducted using R, version 4.1.0 (R Core Team, 2017) with a significant level (α) of 0.05.

Results

Characteristics of the participants

None of the participants presented cardiovascular, respiratory, or metabolic diseases accordingly to PAR-Q questionnaire results. The clinical and demographic characteristics of the participants are depicted in Table 1. No significant associations between age and the main vascular outcomes were observed (i.e., flow-mediated slowing and flow-mediated dilation).

Table 2. Characteristics of the participants (n = 24)

Characteristic	
Age (years)	45.2(19.4)
Height (m)	1.7 (0.1)
Weight (kg)	78.5 (9.3)
Body mass index (kg/m2)	25.7 (2.4)
Waist circumference (m)	0.9 (0.1)
Fat mass (%)	21.3(6.5)
Free fat mass (%)	61.5 (6.7)
bSBP (mmHg)	125.1 (13.6)
bDBP (mmHg)	76.0 (11.2)
HR (b.min-1)	59.9 (7.6)
FMD (%)	6.08 (5.06)
Dbas (mm)	3.99 (0.60)
bPWVbas	
1st Rater	8.63 (0.93)
2nd Rater	8.60 (0.83)
FMS (%)	
1st Rater	-7.01 (4.00)
2nd Rater	_6.57 (3.64)

Data presented as mean (SD). Abbreviations: bSBP: brachial systolic blood pressure; bDBP: brachial diastolic blood pressure; FMD: flow-mediated dilation; Dbas: resting brachial artery diameter; bPWVbas: baseline brachial pulse wave velocity; FMS: flow-mediated slowing;

Inter-rater reproducibility

Intra-and inter-day analyses of FMS showed a good inter-rater reproducibility with an intraclass correlation coefficient (ICC) of 0.81 (95% CI: 0.56 to 0.91) and an ICC of 0.82 (95% CI: 0.57 to 0.92), respectively (Table 2).

Table 2. Inter-rater reproducibility statistics

			95% ICC Confidence Interval	
Variables	CV	ICC	Lower bound	Upper bound
FMS Intra-day (m/s)				
First Measure	19	0.81	0.57	0.92
Second Measure	18	0.86	0.67	0.94
FMS Inter-day (m/s)				
Measure	16	0.82	0.57	0.92
bPWVmin Intra-day (m/s)				
First Measure	2	0.94	0.87	0.98
Second Measure	1	0.97	0.94	0.99
bPWVmin Inter-day (m/s)				
Measure	1	0.98	0.95	0.99
bPWVbas Intra-day (m/s)				
First Measure	1	0.96	0.91	0.98
Second Measure	1	0.98	0.95	0.99
bPWVbas Inter-day (m/s)				
Measure	0	1.00	0.99	1.00

Intraclass correlation coefficients (ICC) and coefficients of variation (CV) were calculated over the two measurements. Abbreviations: FMS: flow-mediated slowing PWVmin: minimum value of pulse wave velocity bPWVbas: baseline brachial pulse wave velocity

The Bland-Altman plot between raters for intra-day FMS analyses showed a mean (bias) difference of -0.44 % (SD = 3.07) for the first time-point analysis, and -0.69% (SD = 2.93) for the second time-point analysis, both time-point analyses presented narrowed 95% CI for bias estimates (-1.74 to 0.85 and -1.93 to 0.54, respectively). One participant did not fall within the 95% limits of agreement (LOA) (Figure 1). In terms of the inter-day analyses, Bland-Altman plots for inter-raters agreement showed a bias of -0.25% (SD = 2.17) with a narrow 95% CI for bias (-1.17 to 0.65). Two participants didn´t fall within the 95% LOA (Figure 1).

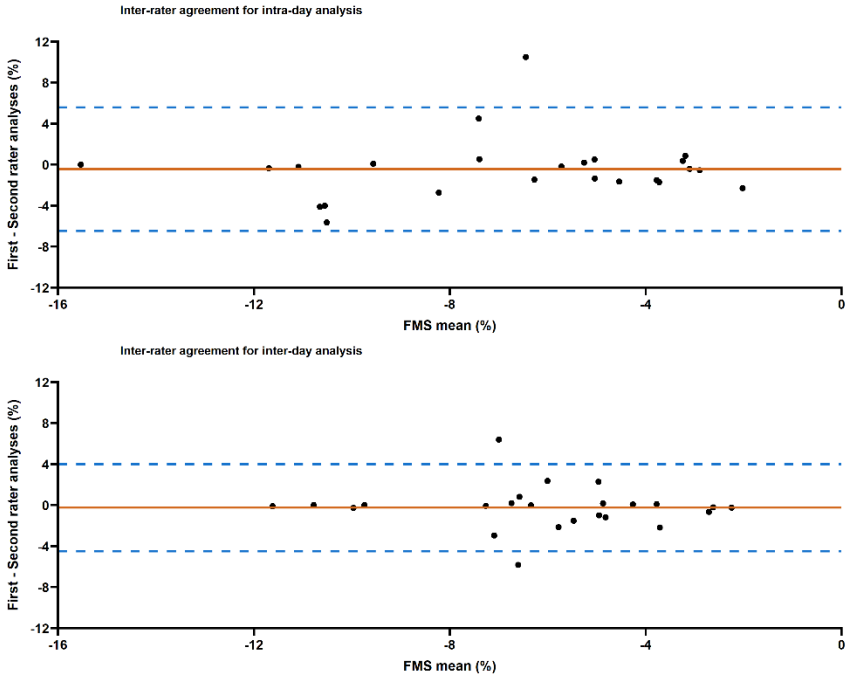

Figure 2. Inter-rater Bland-Altman plot for intra (top) and inter-day (bottom) FMS. Y-axis represents the difference between the first and second measures; the dashed blue lines correspond to the 95% LOA and the red line represents the mean of the differences. For the intra-day, the lower LOA was - 6.47 % (95% CI: - 8.71 to – 4.22); and the upper LOA was 5.57% (95% CI: 3.32 to 7.82). For the inter-day, the lower LOA was – 4.51 % (95% CI: -6.10 to -2.92); and the upper LOA was 3.99% (95% CI: 2.41 to 5.58).

Intra-rater reproducibility

The intra-rater reproducibility statistics are displayed in Table 3. The first rater on intra-day repeated analyses of FMS showed an excellent reproducibility with an ICC of 0.95 (95% CI: 0.89 to 0.97) and with a CV < 15%. Second rater showed a good reproducibility with an ICC of 0.87 (95% CI: 0.71 to 0.94), and with CV = 20%.

Table 3. Intra-rater reproducibility statistics

			95% ICC Confidence Interval	
Variables	CV	ICC	Lower bound	Upper bound
FMS Intra-day (m/s)				
1st Rater	13	0.95	0.89	0.98
2nd Rater	24	0.89	0.72	0.95
FMS Inter-day (m/s)				
1st Rater	21	0.78	0.51	0.91
2nd Rater	36	0.53	-0.07	0.80
bPWVmin Intra-day (m/s)				
1st Rater	3	0.89	0.70	0.96
2st Rater	3	0.90	0.72	0.96
bPWVmin Inter-day (m/s)				
1st Rater	3	0.87	0.70	0.94
2st Rater	3	0.84	0.63	0.93
bPWVbas Intra-day (m/s)				
1st Rater	4	0.89	0.67	0.96
2st Rater	3	0.92	0.71	0.97
bPWVbas Inter-day (m/s)				
1st Rater	4	0.82	0.58	0.92
2st Rater	4	0.73	0.37	0.88

Intraclass correlation coefficients (ICC) and coefficients of variation (CV) were calculated over the two measurements. Abbreviations: FMS: flow-mediated slowing PWVmin: minimum value of pulse wave velocity bPWVbas: brachial pulse wave velocity baseline

The Bland-Altman plot for the first rater on intra-day FMS analyses showed a bias of 0.39 % (SD = 1.66) with a narrow 95% CI (-0.30 to 1.09). There was no evidence of proportional bias, and two participants did not fall within the 95% LOA (Figure 2). The second rater exhibited a bias of 0.14% (SD = 2.69) with a narrow 95% CI (-0.99 to 1.28). Only one participant did not fall within the 95% LOA (Figure 2).

In inter-day FMS analyses, the first rater presented a bias of -0.80% (SD = 2.83) with a narrow 95% CI (-1.99 to 0.39). The second rater showed a mean of -0.61% (SD = 3.96) with a narrow 95% CI (-2.28 to 1.06). Both raters had two participants that did not fall within the 95% LOA (Figure 3).

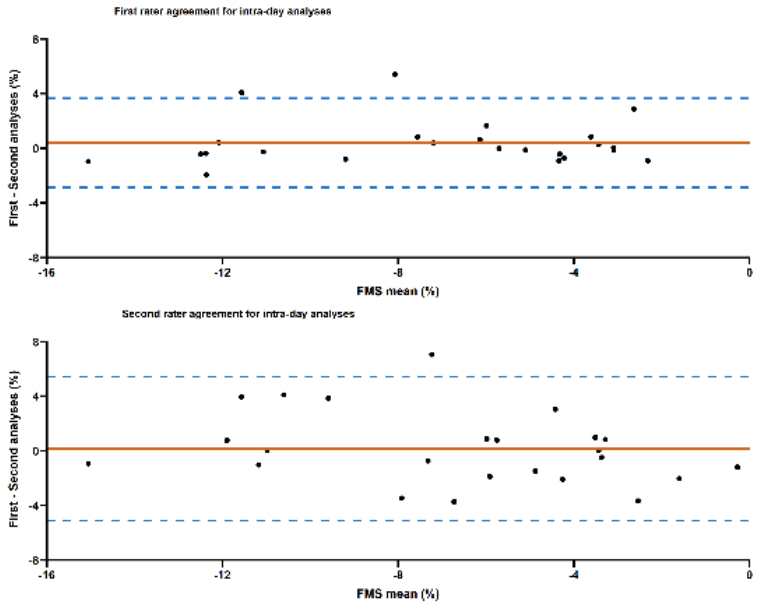

Figure 2. Intra-rater Bland-Altman plots for 1st Rater (top) and 2nd Rater (bottom) intra-day FMS. Y-axis represents the difference between the first and second measures; the dashed blue lines correspond to the 95% LOA and the red line represents the mean of the differences. For the 1st rater (top), the lower LOA was – 2.85 % (95% CI: -4.07 to -1.64); and the upper LOA was 3.65% (95% CI: 2.43 to 4.86). For the 2nd rater (bottom), the lower LOA was – 5.13 % (95% CI: -7.10 to -3.16); and the upper LOA was 5.43% (95% CI: 3.45 to 7.40).

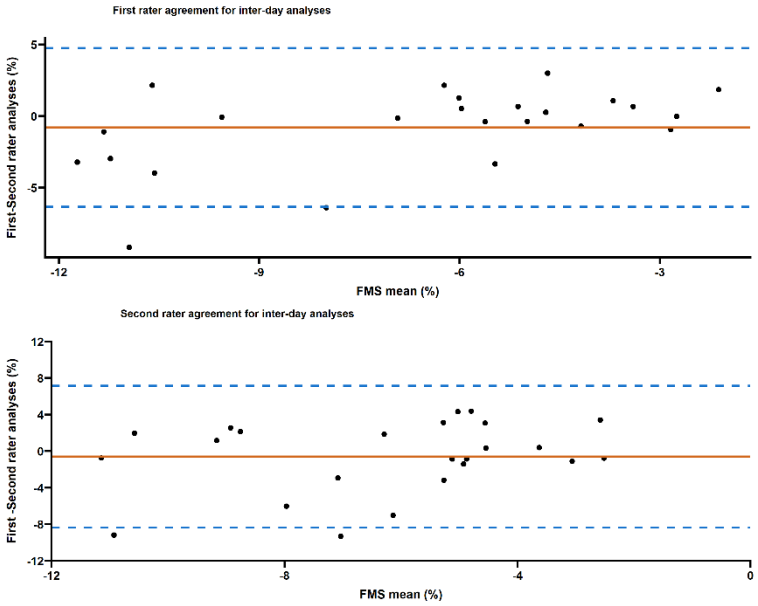

Figure 3. Intra-rater Bland-Altman plots for 1st Rater (top) and 2nd Rater (bottom) inter-day FMS. Y-axis represents the difference between the first and second measures; the dashed blue lines correspond to the 95% LOA and the red line represents the mean of the differences. For the 1st rater (top), the lower LOA was – 6.35 % (95% CI: -8.42 to -4.28); and the upper LOA was 4.75% (95% CI: 2.68 to 6.82). For the 2nd rater (bottom), the lower LOA was – 8.38 % (95% CI: -11.27 to -5.48); and the upper LOA was 7.15% (95% CI: 4.25 to 10.05).

Discussion

This study aimed to examine single and multiple rater reproducibility of FMS using local estimates of bPWV using ultrasound. To the best of our knowledge, this study was the first to show that inter- and intra-rater offline analyses of local FMS are reproducible.

Inter-rater reproducibility of flow-mediated slowing

The inter-rater reproducibility was good to excellent in local FMS off-line analyses on both intra and inter-day measures, with ICCs between raters > 0.80. Although the higher CVs observed between raters in the offline analyses of local FMS (CVs < 20%) may be suggestive of a lack of agreement between or within raters, it can also represent a statistical artifact, as CVs are inflated when the mean of the measurements is close to zero (Pélabon et al., 2020). This was in fact the case on a few participants with less pronounced deceleration of PWV to reactive hyperemia, and resulting in an FMS ~ 0. Furthermore, the inter-rater reproducibility for both PWV_{bas} and PWV_{min} was excellent with CV < 2% and ICCs > 0.90, indicating that the reproducibility of the two parameters by themselves is optimal when they are isolated from the ratio, although they can contribute to the propagation error as suggested by Atkinson & Batterham, (2013b).

Both raters demonstrated a moderate to good intra-day reproducibility in the offline analyses of local FMS, both with CVs below 25%. These findings do not corroborate those by Marôco et al., (2021) who concluded that segmental FMS was not reproducible over intra-day repeated measures (CV > 100%). The use of local PWV to estimate FMS is advantageous as it truly reflects the stiffness response of the brachial artery to reactive hyperemia and reduces both the methodological (e.g., distance measurement) and physiological (e.g., vascular viscous-elastic heterogeneity) confounders inherent to segmental PWV measurements. In the present study, consistent decelerations to reactive hyperemia were observed in local PWV that were not observed in segmental PWV (Marôco et al., 2021), which might lend support that increases in shear-stress NO-mediated vasodilation underpin local but not segmental PWV reductions to reactive

hyperemia. Thus, these confounding factors may explain the equivocal reproducibility of FMS measurements between studies, and may likely extend to other devices used on FMS reproducibility studies.

Intra-rater reproducibility of flow-mediated slowing

Reproducibility was lower in inter-day compared to intraday off-line analyses of local FMS. In fact, one of the raters obtained a CV > 30%. Previous single-rater studies assessing FMS using segmental PWV reported both poor (Marôco et al., 2021) and optimal (Ellins et al., 2016) inter-day reproducibility. In the present study, inter-day reproducibility was higher compared to that reported by Marôco et al., (2021), who reported CV >100%, but lower when compared to that reported by Ellins et al., (2016), who reported CVs < 10%. Alike the findings Ellins et al., (2016) in an arterial segment, intra-rater reproducibility of PWV_{min} and PWV_{bas} was excellent over intra and inter-day off-line analyses of local FMS, and higher than that reported by Marôco et al., (2021).

Limitations

Our study has some important limitations. As we estimated FMS based on local brachial PWV derived from the raw signals of the FMD procedure the reproducibility of FMS depends on the reproducibility of FMD measurements, and thus the recognized limitations of the FMD procedure apply to FMS. Secondly, we did not evaluate endothelium-independent vasodilation in response to sublingual glyceryl trinitrate consequently, we cannot ascertain the contribution of smooth muscle cells to PWV response to reactive hyperemia. Thirdly, we only sampled healthy male individuals. The reproducibility of both measurements of FMD and FMS may be lower in participants with cardiovascular risk factors and chronic diseases (Dick H.J. Thijssen et al., 2019). Moreover, we cannot rule out the possibility of sex differences concerning FMD and FMS reproducibility and different FMS response patterns.

Conclusion

Our study demonstrated that off-line analyses of FMS using raw signals from ultrasonography were reproducible among different raters.

Part 3

Closing Remarks

FMS derived from segmental PWV has been suggested to be useful as a peripheral marker of endothelial function suitable for the evaluation of large-scale populations and early-stage disease cohorts due to its simplicity and apparent superior reproducibility in comparison to FMD (Ellins et al., 2016). However, estimating FMS based on reactive hyperemia-induced changes on local PWV of the brachial artery disputes this rationale. In fact, this methodological approach is only possible if FMD is assessed, and thus it was not unexpected that the intra-rater reproducibility of FMS was identical to the FMD intra-rater reproducibility (CVs < 25%) reported by our group and others. Thus, it may be pertinent to use FMS as a complementary method to FMD, rather than an alternative method.

An important key consideration dictating the utilization of a method in clinical settings and often overlooked in reproducibility studies is the agreement between raters. We found that inter-rater off-line analysis of FMS was optimal, which is in line with previous work that assessed the reproducibility of FMD off-line analysis (Ratcliffe et al., 2017). This suggests that the source of error measurement may not come from offline analysis of both FMD and FMS but rather from conducting/collecting the measurement, hence the need for extensive operator training and adhering to the standardized FMD procedure.

Future research related to the FMS as an alternative measure to the FMD procedure must clarify 1) the reproducibility of FMS estimated using segmental PWV; 2) the underlying mechanisms of the reactive hyperemia induced changes on segmental PWV; 3) and the prognostic value of FMS for cardiovascular diseases and events.

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Supplements

Supplement 1. Intra-day descriptive summary

Variables	Mean	SD	Max	Min	IQR
FMS (%)					
1st Rater	-7.01	4.01	-1.20	-15.54	5.77
2nd Rater	-6.57	3.64	-0.88	-15.53	5.27
FMSabs (%)					
1st Rater	-0.62	0.37	-0.24	-1.47	0.51
2nd Rater	-0.58	0.38	-0.08	-1.47	0.40
betaPWVbas (m/s)					
1st Rater	8.63	0.93	10.63	6.75	1.22
2nd Rater	8.60	0.83	10.67	7.20	0.99
PWVmin (m/s)					
1st Rater	8.00	0.84	9.56	6.41	1.22
2nd Rater	8.03	0.78	9.75	6.99	1.24
bStiffness Overall					
1st Rater	21.39	5.25	33.45	13.40	8.90
2nd Rater	21.67	5.40	33.45	13.40	9.21

Data presented as mean (SD). Abbreviations: FMS: flow-mediated slowing FMSabs: flow-mediated slowing absolute betaPWVbas: baseline beta pulse wave velocity PWVmin: minimum value of pulse wave velocity bStiffness Overall: overall beta stiffness