EXPERT-CONSENSUS AND EVIDENCE-BASED GUIDELINES FOR THE ASSESSMENT OF FLOW MEDIATED DILATION (FMD) IN HUMANS

DICK HJ THIJSSEN[1,2], ROSA MARIA BRUNO[3], ANKE VAN MIL[1,2], SOPHIE HOLDER[1], FRANCESCO FAITA[4], ARNO GREYLING[5], PETER ZOCK[5], STEFANO TADDEI[3], JOHN E. DEANFIELD[6], THOMAS LUSCHER[7], DANIEL J GREEN[1,8], LORENZO GHIADONI[3]

[1]*Research Institute for Sport and Exercise Science, Liverpool John Moores University, Liverpool, United Kingdom*
[2]*Radboud Institute for Health Sciences, Department of Physiology*, Radboud University Medical Center, Nijmegen, The Netherlands
[3]*Department of Clinical and Experimental Medicine*, University of Pisa, Pisa, Italy
[4]*Institute of Clinical Physiology*, National Research Council, Pisa, Italy
[5]Unilever R&D Vlaardingen, Vlaardingen, The Netherlands
[6] *Institute of Cardiovascular Sciences, University College of London, London, United Kingdom*
[7] London University Heart Center, Royal Brompton and Harefield Hospitals, *London, United Kingdom*
[8]*School of Sport Science, Exercise and Health,* The University of Western Australia, Crawley, Western Australia, Australia

SHORT TITLE: Guidelines for flow-mediated dilation

Author for correspondence:

Rosa Maria Bruno

Department of Clinical and Experimental Medicine, University of Pisa

c/o Ospedale Santa Chiara, Building #8

Via Roma 67, 56124 Pisa, Italy

tel: +39 050 992914 / 050 992711

fax: +39 050 992409

email: rosamaria.bruno@unipi.it

ABSTRACT

Endothelial dysfunction is involved in the development of atherosclerosis, which precedes asymptomatic structural vascular alterations as well as clinical manifestations of cardiovascular disease (CVD). Endothelial function can be assessed non-invasively using the flow-mediated dilation (FMD) technique. FMD represents an endothelium-dependent, largely nitric oxide (NO)-mediated dilatation of conduit arteries in response to an imposed increase in blood flow and shear stress. FMD is affected by cardiovascular (CV) risk factors, relates to coronary artery endothelial function, and independently predicts CVD outcome. Accordingly, FMD is a tool for examining the pathophysiology of CVD and possibly identifying subjects at increased risk for future CV events. Moreover, it has merit in examining the acute and long-term impact of physiological and pharmacological interventions in humans.

Despite concerns about its reproducibility, the available evidence shows that highly reliable FMD measurements can be achieved when specialised laboratories follow standardised protocols. For this purpose, updated expert-consensus guidelines for the performance of FMD are presented, which are based on critical appraisal of novel technical approaches, development of analysis software, and studies exploring the physiological principles underlying the technique. Uniformity in FMD performance will *i.* improve comparability between studies, *ii.* contribute to construction of reference values, and *iii.* offer an easy accessible and early marker of atherosclerosis that could complement clinical symptoms of structural arterial disease and facilitate early diagnosis and prediction of CVD outcomes.

KEYWORDS: flow-mediated dilation, methodology, cardiovascular disease, brachial artery, vascular function

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

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

appraisal of novel technical advances, development of analysis software, and studies exploring physiological principles underlying the technique.

1. Vascular reactivity tests

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

2. Clinical value of FMD

2.1 Association with CVD prognosis

Impaired FMD has been associated with conditions predisposing to atherosclerosis and CVD, representing an early step in developing subclinical target organ damage and late clinical events.[18] Brachial FMD is associated with carotid intima-media thickness progression in a population free of CVD[7] and in hypertensive, postmenopausal women.[19] In a 3-year follow-

up study in hypertensive patients, FMD predicted target organ damage progression (including carotid intima-media thickness, pulse wave velocity, albuminuria and left ventricular hypertrophy), even after adjustment for known risk factors.[20] Furthermore, impaired FMD represents an independent predictor of in-stent stenosis after single-vessel coronary interventions.[21]

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

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

2.2 Incremental value of FMD over traditional cardiovascular risk factors.

FMD has failed to demonstrate an added value to classical CV risk factors in terms of discrimination and net reclassification.[27] In the Multi-Ethnic Study of Atherosclerosis (MESA) cohort of intermediate risk individuals, FMD did not increase discriminative power or net reclassification index of risk compared to the Framingham Risk Score.[28] However, the low reproducibility of the FMD in the MESA study, in which the intra-class correlation coefficient was 0.54[28] (worse than other studies in healthy volunteers [29, 30] and CVD patients[30, 31]), may have contributed to these negative findings. Alternatively, FMD may

simply reflect compound risk burden that impacts upon vessel function and hence may not provide incremental risk prediction. A recent position paper by the *European Society of Cardiology* Working Group on Peripheral Circulation[32] concluded that FMD is principally a valuable research tool. Moreover, authors stated that, partly because poor standardisation between laboratories and lack of guideline adherence, FMD is currently not recommended for the assessment of CV risk, with evidence grading remaining at III, in accordance with other guidelines.[33, 34]

2.3 Clinical value of long-term changes in FMD.

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

2.4 Potential value of short-term changes in FMD.

Many studies have examined short-term changes in FMD, ranging from a few hours, to several days, and up to months. In these randomised trials, FMD was selected as the primary

outcome measure to investigate the potential protective impact of (non-)pharmacological therapies.[36, 40-42] Increase in FMD in response to (non-)pharmacological substances may translate to long-term improvements in endothelial function and protection against atherosclerosis and/or CVD. The relative simplicity of the design and the ability to perform reproducible, repeated measurements explain the popularity of examining changes in FMD in these studies.[14]

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

3. Ultrasound assessment: technical requirements and common mistakes

3.1 Diameter and velocity assessment.

FMD is typically examined in the brachial artery (diameter 3-5 mm). Longitudinal images of the brachial artery are taken using high-resolution B-mode ultrasound, usually with an ultrasound probe of 7.5-12 MHz. Studies have also examined smaller (e.g. radial artery: diameter 1.5-3 mm) or larger sized arteries (e.g. superficial femoral artery: diameter 5-7 mm, popliteal artery: diameter 4-6 mm). Changes in resolution may be required to optimize B-mode images in arteries that lie at differing depths. A key challenge with B-mode imaging is to identify clear vascular boundaries, i.e. the double lines of Pignoli (Figure 2).[44, 45,46] A

reasonable approach involves obtaining an image characterised by a single echogenic layer, representing the brachial artery wall-lumen interface. Since diameter is a key determinant of FMD, the modality of measurement applied may influence results and, under all circumstances, should be kept the same throughout the experiment. Special attention should be paid to perform adequate scanning of the baseline diameter. More specifically, tangential scanning is a common error and results in underestimation of the true brachial artery diameter. Recent technological advances, which adopt an H-shaped probe capturing 2 short-axis and 1 long-axis for automatic probe position correction, may overcome this crucial limitation.[47]

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

Finally, the sample volume used for pulsed-wave Doppler should be considered. Since measurements settings of the width of the sample volume vary between laboratories, and

impact upon the analysis of the velocity signal (see section 3.2), we recommend to maintain consistency in methods within studies, and in particular for repeated tests within subjects.

3.2 Analysis of the velocity signal.

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

a) Shear rate = 8 × mean blood velocity / internal diameter; for large, centred sample volume;

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

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

3.3 Maintaining the optimal image: probe holder versus hand-held.

Another common mistake is inconsistency in scanning the same portion of the artery during repeated measurements, or throughout a single FMD. Therefore, anatomical landmarks should be identified and recorded and/or the distance between the elbow crease and ultrasound probe should be recorded (or photographed). The position of the subject (related to

the arm and hand), but also cuff size and position (see 4.2.1) should be also recorded and standardised.[58]

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

4. Practical considerations, study protocol and data analysis

4.1 Subject preparation and environmental influences.

Subject preparation is important in the valid and reproducible assessment of the FMD, with many studies failing to control for these factors and/or failing to report on their procedures in sufficient detail. Several subject-related factors can influence FMD, including food, alcohol, smoking, supplements, drugs, physical activity, and mental stress.[61-64] While some factors directly impact stimulated NO-release, others, such as acute physical exercise,[62] modify baseline vasomotor tone (and therefore baseline diameter).[4] To minimize their effect, it is strongly recommended that prior to FMD examination, subjects are fasted (>6h), avoid

exercise (>24h), and refrain from caffeine, vitamin C, polyphenols, alcohol and supplements known to affect the cardiovascular system for a consistent period of time (typically>12h) (Table 2). Smokers must refrain from smoking for a standardised period (preferably>6h). When examining patient groups taking drugs, we recommend waiting 4 times the half-life of the drug. If drug intake cannot be avoided, examination should be performed after a consistent and standardized time.[59] Since mental stress affects FMD,[64, 65] testing must be performed in a quiet, temperature-controlled room after a standardised period of supine rest of >10-15 minutes. Acute intense mental stress can cause prolonged (up to 90 min) impairment in FMD[66], but its confounding effect can be hardly controlled or eliminated. Premenopausal women should be examined in a standardised phase of the menstrual cycle, since hormonal changes can affect FMD.[67] Vascular function in humans, including that assessed by FMD,[68] demonstrates a diurnal variation. This implies that the time of the day of FMD should be standardised and reported, especially when performing repeated measures. Other environmental factors that may affect FMD are outdoor temperature,[69] seasonality[70] and air pollution.[71] However, because of practical reasons, these factors should not be considered as limiting when ideal conditions cannot be achieved.

4.2. Study protocol

4.2.1 Protocol: cuff position.

Placement of the occlusion cuff importantly alters the magnitude,[72] duration,[72,73] nature[74] and possibly the clinical relevance[26] of the dilator response. Originally, Celermajer[13] examined brachial artery FMD with the occlusion cuff placed around the forearm, distal to the ultrasound probe (Figure 2). Standardising cuff position is of crucial importance for valid comparisons between studies, since occlusion of a smaller volume (i.e. placement towards the

wrist) or larger volume of tissue (i.e. cuff occlusion around the upper arm) leads to either a markedly attenuated diameter[58] or an enhanced[72,75] shear stress response.

Some have supported the placement of the occlusion cuff above the imaged artery,[73] with the main argument that the larger dilator response[76] achieved by occluding a larger area may improve FMD discriminative power. One analysis found no significant differences in prognostic value between studies that adopted distal *versus* proximal cuff position.[26] Furthermore, some limitations of proximal cuff inflation must be considered. First, scanning the brachial artery during this procedure is challenging, since the artery can collapse and/or tissue movements can worsen image quality. Secondly, the dilator response with proximal cuff position is largely mediated through vasoactive substances other than NO.[61] A meta-analysis[74] found that the dilation in response to distal cuff occlusion is ~70% NO-mediated, whereas proximal cuff placement is only ~30% NO-mediated. These differences between studies are confirmed by direct comparisons of distal *versus* proximal cuff position in the same individual.[73] This is mechanistically important, because several studies specifically aim to study the NO-dependent pathway, given its established importance in the process of atherosclerosis. Thus, strict standardization of distal cuff occlusion (i.e. below the imaged artery) is recommended to ensure maximal dependence of the dilator response on endothelium-derived NO.

4.2.2 Protocol: baseline diameter.

The FMD-response is typically expressed as the relative change in post-hyperaemia diameter from baseline. As a direct consequence and limitation, the FMD is a function of the degree of responsiveness to stimuli, but also of baseline vasomotor tone and structural remodeling. Correct assessment of baseline diameter is therefore of crucial importance and implies the removal of potential factors influencing both baseline diameter and vasomotor tone (see

section 4.1), but also practical (3.1+this section) and statistical procedures (4.4.2+this section) linked to baseline diameter.

Most studies use the pre-inflation diameter to calculate the FMD, in line with the classic approach[13] and previous FMD-guidelines.[58,59,61] It is recommended that pre-inflation diameter should be recorded for 1-minute, with a minimum of 30-seconds.[61] The end-of-ischemia diameter has been also proposed as the baseline diameter for FMD calculation. However, "low-flow" state during distal cuff occlusion may lead to artery constriction, though this phenomenon appears to be evident mostly in the radial[77-79] rather than in the brachial artery.[49,80-82] Conversely, one study showed that brachial artery diameter during cuff inflation was significantly larger than that assessed pre-inflation, leading to a lower FMD, an effect not present in older groups.[83] Based on these between-group differences, we recommend using pre-occlusion diameters as the baseline value so that the differential impacts of cuff occlusion on the baseline artery diameter are avoided.

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

4.2.3 Protocol: duration and magnitude of cuff occlusion.

The original 5-minute duration of cuff occlusion remains the most frequently used protocol. Shorter periods lead to negligible dilation,[89] whilst longer periods lead to larger blood flow and diameter responses, but may be less tolerable for volunteers.[72] Since the nature of dilation changes with longer periods of occlusion,[75] we recommend using a 5-minute occlusion period.

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

4.2.4 Protocol: post-deflation measurements.

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

Endothelial function is increasingly examined in conduit arteries other than the brachial artery. However, the nature of the diameter responses in other arteries may differ from that in

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

4.3 Identification of the peak diameter.

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

4.4. Shear stimulus

4.4.1 Shear stimulus: importance for FMD

Although the terminology of FMD suggests that dilation is flow-mediated, physiological work demonstrates that it is rather mediated through the post-deflation increase in shear stress.[48] Studies that adopted within-subject manipulation of the post-deflation shear stress stimulus revealed that the relation between shear stress and dilation is dose-dependent.[48, 49, 98, 99] This fits with landmark studies[100, 101] demonstrating the importance of shear stress in mediating endothelium-dependent dilation. Pyke and Tschakovsky[49] suggested that the total, rather than peak shear stress, is more important in mediating conduit artery dilation.[49] This

finding was later confirmed by others.[50] These studies show physiological and mechanistic basis for the continuous recording of both conduit artery diameter and velocity (Figure 1).

4.4.2 Shear stimulus: normalization for FMD stimulus

Based on the importance of shear stress as the eliciting stimulus, and also the assumption that within-/between-subject variation in FMD-responses relate to the magnitude of reactive hyperaemia, studies have used different approaches to account for the shear stress stimulus. An early approach involved 'simple' ratio normalization by dividing FMD by shear rate stimulus.[50,81,102] However, this approach tends to violate important statistical assumptions, being (i) the relationship between both parameters is linear, (ii) the intercept for the regression slope of this relationship is zero, (iii) data (including residuals) are normally distributed, (iv) variances are similar between groups and (v) the ratio does not lead to spurious correlations with other variables.[103] Whilst acceptable-to-good relation between the shear rate stimulus and FMD is present *within* subjects,[48-50,89,99,104] studies examining this relation *between* subjects found a weak[95] or absent[105] relation. Whilst this does not invalidate the role of shear stress as the dilator stimulus, it indicates that FMD variability cannot be simply controlled for by ratio normalization.[103]

Studies have also explored other strategies to statistically correct the FMD-response for the shear stress stimulus, such as including shear rate as a covariate in an analysis of covariance (ANCOVA).[106-108] ANCOVA results, however, could be misleading if the covariate (i.e. shear rate), is related to the independent and/or outcome variable (e.g. age, sex, baseline diameter and FMD). However, since calculation of shear rate importantly depends on baseline diameter, shear rate normalization may not be needed if the FMD is scaled properly to baseline diameter using the previously mentioned allometric scaling.[109]

Taken together, it is currently not clear which statistical strategy is preferred to account for the eliciting shear rate stimulus in the FMD response. Ratio-normalization seems statistically flawed, whilst too many questions remain present around validity, practicality and potential clinical importance of statistical correction for the shear rate stimulus. It is recommended to report the relevant shear rate stimulus, i.e. shear area-under-the-curve up to the peak diameter. Reporting the shear stress stimulus may also be clinically relevant because the post-occlusion hyperaemia is strongly related to CV risk, and may have prognostic value for future CV events.[110-113]

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

Early FMD research relied on a manual approach to assess diameter change through visual inspection and calipers placement.[13,114] This method is highly operator-dependent, carries risk of observer error, and is time-consuming.[115-119] Recent years, software systems for automatic diameter measurement have been developed. This represents a time-efficient approach, and has limited operator-related errors and bias. More importantly, intra-observer variation is significantly lower with automated analysis compared to the classic manual technique.[46, 115, 116, 118-120]

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

assessments within a given frame, whilst recent speckle tracking systems have also overcome the spatial resolution limitation.[121]

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

An additional advantage of automated systems is their ability to use both ECG-gated and non-gated ultrasound images without affecting the FMD results.[96] This can help to keep the experimental setup as simple as possible, for example by adopting a cheaper entry-level ultrasound device without ECG synchronization capability. Software systems for automatic FMD evaluation should be usable and user-friendly, but also should provide options to view and record different parameters (i.e. shear rate). Furthermore, the high throughput of data, ease of use and other technical features (e.g. real-time feedback, overlay of edge detector output with B-Mode) may help adequate training of the operator. Finally, any new automatic system must be validated in appropriate populations before introducing its use in laboratories.[45, 122]

4.6.Summary and future directions.

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

5. Reproducibility of FMD measurements

Several studies have examined FMD reproducibility. However, these have been performed with widely varying adherence to FMD-guidelines. After pooling the results of 7 centers that strictly followed FMD-guidelines, including optimal training of operators and monitoring, Ghiadoni *et al.* found that FMD in healthy volunteers is highly reproducible among centers (coefficient of variation 11.6-16.1%).[29] Data from the dal-VESSEL trial also showed reproducible results in 19 laboratories after standardised training in patients with CVD (coefficient of variation 15.8-17.5%).[31] More recently, Greyling and colleagues designed a tool to evaluate adherence to FMD expert consensus guidelines.[123] Subsequently, a meta-analysis was performed to assess the relation between degree of adherence and reproducibility of the FMD.[60] Not surprisingly, stricter adherence to guidelines was related with markedly less measurement error in brachial artery FMD. This work highlights that strictly following contemporary guidelines leads to highly reproducible results.

5.1 Factors influencing reproducibility: group and methodology differences

Guidelines for FMD measurement cannot cover and eliminate all sources of measurement variations and error. Understanding of these factors is important for designing studies on FMD (e.g. sample size calculations). Van Mil *et al*. recently performed analysis of 672 repeated FMD measurements that were collected after strictly following contemporary guidelines.[30] High reproducibility was found in healthy subjects (9.3%), whilst regression models several factors that contributed to variations in reproducibility (see below).

Subject characteristics. Subjects with older age, hypertension, or dyslipidaemia show a larger variability in FMD.[29-31] This supports earlier findings in that CV risk factors impair FMD reproducibility.[124] An explanation for the larger variation in high-risk subjects may relate to the lower baseline FMD% typically observed in these populations.[58, 125, 126] Indeed, Van Mil *et al*. demonstrated that a lower FMD% explained part of the higher variability in FMD with older age and dyslipidaemia.[124] Differences in vascular structure and compliance may also contribute to this higher variability[127] and finally, not all subjects may be equally susceptible to the effects of CV risk factors at the endothelial level.

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

Methodological factors (lab experience). Possibly the most important methodological source of variability in the FMD is proper operator training. Guidance and training by expert colleagues seems crucial, since lower variability is present in more experienced laboratories.[30] Although the need of experience in scanning is mentioned by previous guidelines,[58, 59, 61] it is difficult to quantify "proper training". No study specifically aimed at quantifying the appropriate duration of the training, like what was done for carotid intima-media thickness.[128] Corretti *et al*. suggested that >100 scans/year are required to maintain competency.[59] However, there was no mention of the training required before performing FMD measurements. Two independent consortia adopted a formal training for sonographers: sonographers were qualified for measurements when the CV for repeated scans were <2% for brachial artery diameter and <15% for FMD.[29,31] Since FMD variability seems larger in individuals with CV risk,[29,31] operator training might be longer depending on the study population enrolled. Both aforementioned studies showed excellent reproducibility among centres, suggesting that specialised training is feasible and successful. We therefore recommend that all studies on FMD should report details on preparation and training of the sonographers, along with the observed variability in their FMD measurements.

6. Endothelium-independent dilation

6.1 Pathophysiology and clinical relevance

The degree of FMD can also be influenced by the functionality of vascular smooth muscle (VSM) cells.[129] Any functional defect in VSM cells reduces their capacity to respond to NO, whilst a compensatory increased VSM response may be present in endothelial dysfunction.[130, 131] In any case, it is recommended that the studies on endothelium-dependent vasodilation also determine the extent of any coexistent endothelium-independent dysfunction. Although

this recommendation was already present in previous FMD-guidelines,[59] a standardised protocol has not been clearly defined yet.

Clinical determinants of VSM function are not fully understood. In a large Japanese cohort, brachial artery dilation to sublingual glyceryl trinitrate (GTN) was correlated with most classical CV risk factors and represented an independent determinant of FMD.[132] Conversely, a meta-analysis reported micro-, but not macrovascular, impairment of VSM in type 2 diabetic patients.[133] Since the brachial artery was studied in only 12 out of the 31 studies, this subgroup analysis is likely underpowered. Furthermore, some studies found a relation between brachial artery GTN-responses and lack of nocturnal blood pressure fall[134], coronary artery calcium,[135] and microalbuminuria.[136] Although GTN-responses show a relationship with Framingham Risk score,[132] only few prospective studies investigated the relation between this response and CVD events.[137, 138] Thus, the prognostic value of the GTN-response remains mostly inconclusive.[137, 138] Finally, the doses typically used in these studies induce near-maximal dilation,[72] hence the GTN-responses may be a structural index rather than a functional one.

6.2 Dose, technique, and administration of exogenous nitrates

Many research centers use sublingual GTN 400 mcg, the lowest marketed dose in many countries. Administration of GTN via a sublingual spray leads to a larger and faster response than by tablet administration.[139] Lower dosages (25 mcg) of GTN were later introduced[140] and recommended in the 2005 ESH-statement.[141] The rationale for lower dosages is the induction of less extreme dilatation, a faster return to baseline diameter (~20-min), and lower risk of side effects. In healthy volunteers, 8-35 mcg GTN is estimated to induce a 4%-10% dilation, without changes in blood pressure and heart rate.[132, 142] Furthermore, 25-mcg GTN

did not cause any significant increase in sympathetic signalling directed to muscle vasculature, evaluated by microneurography.[143] It has been suggested that higher GTN doses might be useful to assess maximum vasodilating capacity, thus providing information about structural alterations. However, maximal vasodilation cannot conceivably be obtained in these conditions because of counter-regulatory sympathetic vasoconstriction induced by the high doses of GTN. Although a low-dose GTN is recommended, this approach may not always be feasible, since low-dose GTN for oral use is not commercially available for most countries.

GTN should be administered >10-min after FMD testing to ensure return to baseline diameter. Furthermore, continuous diameter monitoring is recommended, because the timing of peak dilatation varies between subjects.[144] The peak vasodilation response to GTN usually occurs between 4-/5-min, both with high[144, 145] and low GTN doses.[140] Therefore, monitoring should be performed >5-min after GTN administration.

7. Conclusions

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

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FIGURE LEGENDS

FIGURE 1. A: Schematic presentation of diameter and shear stress (or rate) responses before and after a 5-minute ischaemic stimulus applied distally to the forearm.

B: representative diameter (upper panel) and velocity (lower panel) data from a healthy person before and after the 5-minute ischaemic stimulus.

C: representative diameter (upper panel) and velocity (lower panel) data from a person with endothelial dysfunction before and after the 5-minute ischaemic stimulus.

FIGURE 2. Experimental set-up for the performance of the brachial artery FMD.

A: Position of the cuff and ultrasound probe placed on the upper arm.

B: Screen shot of a representative B-mode image of the diameter and Doppler blood flow velocity.

C: Wall tracking software to perform (semi)automated analysis.

D: Output generated by the analysis software to enable calculation of the FMD.