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Abstract—Developed in 1992, the flow-mediated dilation test is now the most commonly used noninvasive assessment of vascular endothelial function in humans. Since its inception, scientists have refined their understanding of the physiology, analysis, and interpretation of this measurement. Recently, a significant growth of knowledge has added to our understanding and implementation of this clinically relevant research methodology. Therefore, this tutorial provides timely insight into recent advances and practical information related to the ultrasonic assessment of vascular endothelial function in humans. (*Hypertension*. 2010;55:1075-1085.)

Key Words: FMD ∎ endothelial function ∎ reactive hyperemia ∎ shear stress

This tutorial provides an easy-to-follow reference for researchers interested in performing flow-mediated dilation (FMD) testing for the first time, but also, because of the numerous recent advancements in this field, an important and timely updated summary of current practices for established researchers in the area. Because vascular endothelial dysfunction represents an initial step toward hypertension and cardiovascular disease, the accurate assessment of vascular endothelial function is an essential tool that will assist in our understanding of the etiology of these vascular-related diseases and determine the efficacy of therapeutic treatments that target vascular health. Beyond a short introduction of the history and physiology behind the ultrasonic assessment of FMD, there follows comprehensive, evidence-based, technical, and interpretive strategies for performing the ultrasonic assessment of endothelial function in humans.

Historical Perspective

In 1992, Celermajer et al[1] developed the FMD technique as a noninvasive method to measure vascular endothelial function. Since this time, the ultrasonic assessment of FMD in response to occlusion-induced hyperemia has been established as a reliable, noninvasive measurement of endothelial function[2] and has been documented to correlate with invasively assessed endothelial function in the coronary arteries.[3] In an effort to standardize this measurement among investigators, in 2002 Corretti et al[4] published the initial guidelines for the ultrasonic assessment of FMD of the brachial artery, which, to date, have been referenced >1000 times. Since then, an ongoing effort has been made to adapt the original methodology introduced by Celermajer et al,[1] to a more robust assessment of a true NO-dependent measurement of vascular endothelial function. In 2005, a meta-analysis was conducted on 250 studies that used the measurement of FMD and revealed that technical aspects of the measurement (ie, occlusion location and duration) may explain the differences in FMD observed among studies.[5] At this time, Deanfield et al[6] published their recommendations for global endothelial function testing with a specific section highlighting the noninvasive FMD technique. Most recently, Pyke and Tschakovsky[7] provided an update to the guidelines presented by Corretti et al[4] that specifically targeted the issue of the shear stress stimulus and have provided important recommendations that are now common practice for FMD testing. Despite these considerable advancements in the understanding and application of the FMD technique, this comprehensive tutorial offers up-to-date technical instructions for the performance and interpretation of FMD.

Endothelium

The endothelium plays multiple pathological and physiological roles, including the regulation of smooth muscle tone, control of thrombosis, inhibition of leukocyte and platelet cell adhesion, and promotion of intra-arterial permeability.[8–10] In addition, there are numerous vasoactive substances released from the endothelium, including prostacyclins, endothelins, endothelial cell growth factors, interleukins, plasminogen inhibitors, and NO. The latter is, perhaps, the major mediator of vasodilation[11] and has, thus, been intensely studied since its discovery in 1980.[12] After ≈30 years of NO-related research, reduced NO bioavailability has become synonymous with the condition broadly described as "endothelial dysfunction."[13] In addition to being proposed as the primary etiology of atherosclerosis,[14] endothelial dysfunction is the earliest identifiable event in the process of atherosclerotic cardiovascular disease, the leading cause of morbidity and

From the Georgia Prevention Institute (R.A.H.), Medical College of Georgia, Augusta, Ga; College of Osteopathic Medicine (S.K.N.), Touro University, Henderson, Nev; Division of Geriatrics, Department of Medicine (D.W.W., R.S.R.) and Department of Exercise and Sport Science University of Utah (D.W.W., R.S.R.), Salt Lake City, Utah; Geriatric Research, Education, and Clinical Center (D.W.W., R.S.R.), Salt Lake City Veterans' Affairs Medical Center, Salt Lake City, Utah.

Correspondence to Ryan A. Harris, 1120 15th St, Medical College of Georgia, Georgia Prevention Institute, Augusta, GA 30912. E-mail ryharris@mcg.edu

mortality in the United States.[15] It follows that the assessment of endothelial function has become an area of considerable interest to the medical and research communities.

Flow-Mediated Dilation

When measured appropriately, the assessment of endothelial function via FMD has been proposed to represent a functional bioassay for endothelium-derived NO bioavailability in humans.[13] During an FMD test, vasodilation occurs after an acute increase in blood flow, typically induced via circulatory arrest in the arm (suprasystolic cuff occlusion) for a period of time. Specifically, this hyperemia increases laminar shear forces parallel to the long axis of the vessel,[16] which is transduced via luminal mechanoreceptors to the endothelial cell. This event increases G-protein expression of phosphokinase A, signaling an increase of endothelial NO synthase activity, which catalyzes the conversion of L-arginine to NO.[17] NO then diffuses into the tunica media, where it activates soluble guanylate cyclase, which converts GTP into GMP to induce relaxation of the smooth muscle and subsequent vasodilation. In its traditional form, the increase in arterial diameter, as a consequence of the reactive hyperemia, is compared with the baseline diameter and expressed simply as a percentage of this baseline diameter (% FMD). Despite this intuitive and "attractive" link between FMD testing and NO bioavailability, it should be noted that vessel type and size may influence the relative contribution of NO,[18] and there is still some debate about this in the literature with data both for[13,19,20] and against the concept that vasodilation mediated by the endothelium is predominantly a consequence of NO.[21,22]

Measurement of FMD: The Essential Elements

Appropriate Ultrasound Technology

FMD assessed by Doppler ultrasound has emerged as the most popular clinical research method of assessing vascular endothelial function, likely because of the relatively simple methodology and noninvasive nature (Figure 1). However, the appropriate ultrasound equipment, in conjunction with the high level of skill required, is essential for accurate and reliable measurements, as detailed below.

High-Resolution, Multifrequency Linear Ultrasound Doppler Probe

The accurate measurement of FMD is highly dependent on identification of a defined arterial wall, which requires a highly resolved ultrasound image. Each ultrasound probe is classified according to a frequency range in megahertz, which is inversely proportional to the depth of optimal imaging. Figure 2 demonstrates the quality of B-mode images using different frequency probes in the brachial artery at a depth of ≈2 cm. It is clear that. for superficial vessels, a 10- to 14-MHz linear array probe, currently considered "high resolution," is optimum. However, as recommended previously,[4] it is also apparent that a much lower frequency probe will allow the detection of changes in diameter in all but the most superficial of vessels.

Duplex Mode

Duplex mode allows the simultaneous acquisition of B-mode and Doppler for the determination of vessel diameter and blood velocity, respectively. These collective measurements enable the calculation of shear rate (see below) for any given time period from which the integral of shear across time (ie, shear rate area under the curve [AUC]) may be calculated. This is a key advantage of Duplex scanning, because it is the cumulative exposure to shear experienced by the artery that is proposed to be the major stimulus for the FMD response.[23]

Angle Steer and Insonation Angle Correction

With a blood vessel that runs parallel to the surface of the skin (eg, brachial artery), the simple placement of a high-frequency linear ultrasound probe has the potential to yield an excellent image, because the ultrasound beam will bisect the vessel at 90°. However, this is the worst-case scenario for the Doppler assessment of blood velocity, because the accuracy of this measurement technique varies from minimal error at 0° (parallel with the blood flow) to virtually no signal (0 velocity) when the beam bisects the artery at 90° (the optimum angle for imaging). Some compromising of image quality by rocking the transducer (probe) up on one end more than the other (known as heel/toe adjustments) will make the vessel appear to run diagonally across the monitor and bring the Doppler beam to an angle of <90°. Even with excellent technique, to actually attain the accepted insonation angle of ≤60° in the brachial artery is certainly challenging if not impossible. This issue is resolved through use of "angle steer," achieved by asynchronous firing of the phased array of pizo-electric crystals that transmit and receive the Doppler signal. With the beam "steered" left or right ≈20° to 30°, an insonation angle of 60° can be achieved with far less heel/toe movements of the probe and sacrifice of image quality.

Because angle steer is not always available, there have been a significant number of studies that have settled for an insonation of >60°.[24–26] However, it should be noted that a 60° angle of insonation is, in fact, the "best of the worst" angles that should be acceptable, and although this value itself introduces some error, this error is still far less than higher degrees of insonation.[27–29] The significant impact of the angle of insonation determination of blood velocity and the subsequent calculation of blood flow may be seen in the Doppler shift equation:

$$(1) \qquad f_D = 2f_o \frac{v}{c} \cos(\alpha)$$

where f_D is the Doppler shift of the reflected ultrasound, f_o is the transmitted frequency, v is the blood velocity, c is the sound velocity in tissue, and α is the insonation angle between the ultrasound beam and the velocity vector. To illustrate the impact of insonation angle on the measurement of blood velocity, Table 1 displays the velocity, blood flow, and subsequent error among different angles of insonation studied sequentially in 5 individuals. The velocities associated with 70° and 80° angles are significantly ($P<0.05$) elevated when compared with the standard 60° insonation angle; however, only the blood flow calculated from an insonation angle of 80° is significantly augmented when
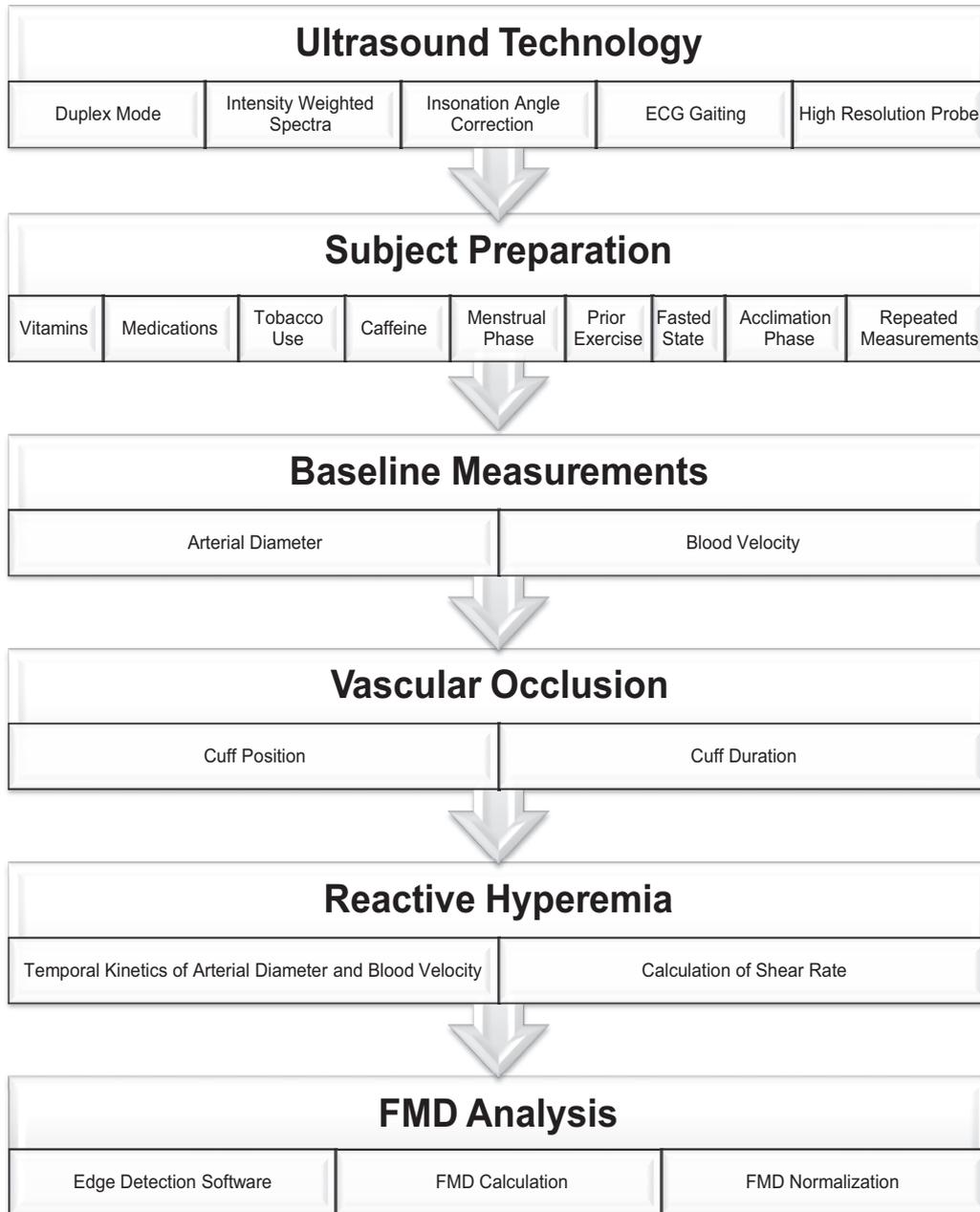
Ultrasound Technology

Duplex Mode	Intensity Weighted Spectra	Insonation Angle Correction	ECG Gaiting	High Resolution Probe

Subject Preparation

Vitamins	Medications	Tobacco Use	Caffeine	Menstrual Phase	Prior Exercise	Fasted State	Acclimation Phase	Repeated Measurements

Baseline Measurements

Arterial Diameter	Blood Velocity

Vascular Occlusion

Cuff Position	Cuff Duration

Reactive Hyperemia

Temporal Kinetics of Arterial Diameter and Blood Velocity	Calculation of Shear Rate

FMD Analysis

Edge Detection Software	FMD Calculation	FMD Normalization

Figure 1. Schematic of the essential elements for the ultrasound assessment of FMD.

compared with 60°. Although an insonation angle of 60° is recommended, the greatest angle typically accepted in literature is 70°.[26]

ECG Gating

Depending on pulse pressure and vascular stiffness, arterial diameter may vary quite considerably across a single cardiac cycle.[30] In some subjects, the change in diameter may be as much as 1 mm, which, if unaccounted for, may completely confound the assessment of FMD. Most ultrasound Doppler systems have an integrated ECG facilitating the assessment of diameter according to the cardiac cycle (eg, end diastole). However, if this feature is not available on the ultrasound system itself, an external ECG can be used to trigger an external image capture/analysis system.[31]

Intensity-Weighted Velocity Calculations

The simplest method of assessing blood velocity uses the outer envelope of the Doppler spectra to determine mean peak velocity, whereas the slightly more complex approach integrates the area under this envelope to calculate mean peak velocity. However, neither of these calculations accurately reflects the complex and varying range of velocities and their relative distributions within the Doppler spectra. Therefore, to accurately assess blood velocity it is recommended that intensity-weighted calculations of the time-averaged mean be performed to most accurately reflect the contribution from red cells moving at differing speeds within the vessel. Basing velocity measurements on the peak envelope of the Doppler spectra will not only overestimate the actual blood velocity but will also yield subsequent calculations of shear profiles
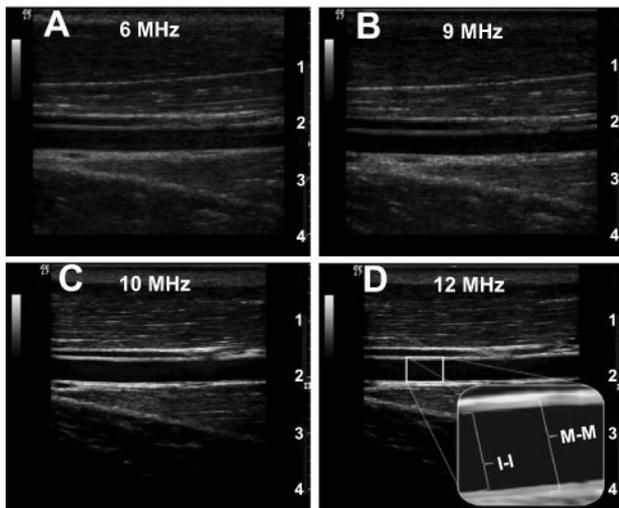
Figure 2. The image quality of B-mode images using different frequency linear probes. A, 6 MHz; B, 9 MHz; C, 10 MHz; and D, 12 MHz. The magnification illustrates both the intima to intima (I-I) and the media to media (M-M) interfaces. Note that although there is definitely a visible layer among all of the images above, using at least a 10-MHz probe offers a very clear identification of the endothelium.

and blood flows that are inaccurate. If consistently used throughout the investigation, the peak velocity envelope approaches do offer a surrogate for true mean velocity measures, although results may conflict with others in the literature using intensity-weighted velocity calculations.[32] In summary, an ultrasound system offering Duplex mode, angle steer and insonation angle correction, intensity-weighted spectra measurements, ECG monitoring, and a high-resolution linear array probe is optimal.

Subject Preparation
To ensure an accurate measurement of FMD, there are several subject-specific factors worth consideration.

Vitamin Supplementation
Just as the in vivo pro-oxidant and antioxidant balance plays a clear role in vascular endothelial function,[33,34] there is direct evidence of a reduction in circulating free radicals after oral antioxidant supplementation (vitamin C, vitamin E, and α-lipoic acid).[33] In addition, the intra-arterial administration of ascorbic acid has been documented to augment FMD.[35]

Therefore, subjects should abstain from vitamin supplementation for ≤ 72 hours before FMD assessment. Although more difficult to control, it should be noted that a diet high in naturally occurring antioxidants may also influence the results of an FMD study.[36]

Medications
Because many medications have both direct and indirect vascular effects, if possible, subjects should refrain from taking all medications for ≥ 4 half-lives of the drug before FMD measurements.[4] In particular, special attention should be paid to medications that target the cardiovascular system (ie, β-blockers, nitrates, and calcium channel blockers), and although cessation of these medications may not be feasible, their potential to confound the results should at the very least be recognized and documented. On the basis of the half-life of nonsteroidal anti-inflammatory agents and aspirin, it is recommended that these be discontinued 1 and 3 days, respectively, before an FMD measurement.

Tobacco Use
Smoking is a classic modifiable risk factor of cardiovascular disease that has been documented to attenuate endothelial function.[37] In addition, even the exposure to second-hand smoke has been shown to attenuate FMD.[38] Thus, it is recommended that subjects refrain from both smoking and smoke exposure for ≥ 12 hours before FMD measurements.

Caffeine
Although there are other pharmacologically active beverages, coffee is the most common source of caffeine. Not only does caffeine inhibit soluble guanylate cyclase, a step in the NO-mediated process that results in vasodilation,[39] caffeinated coffee has been documented to attenuate FMD.[40] Accordingly, caffeine ingestion should be avoided for ≥ 12 hours before FMD testing.

Menstrual Phase
The increased endogenous production of estrogen, concurrently with progesterone, across the menstrual cycle has been documented to increase endothelial NO synthase activity[41] and antioxidant capacity[42] in both human and animal models, thus potentially influencing the vasodilatory response. Consequently, when studying premenopausal women, measurements should be performed at the same time of the menstrual cycle. To minimize the impact to these hormonal changes or

Table 1. Evidence of Alterations In Blood Velocity and Blood Flow as a Consequence of Insonation Angle

Insonation Angle	Velocity, cm/s	Blood Flow, mL/min	% Δ From 60°	Theoretical FMD, %	Theoretical Shear, s⁻¹	Theoretical FMD/Shear
40°	3.52±0.46	24.8±8.3	−49	7.0	33.52	0.21
50°	4.24±0.57	29.5±9.9	−25	7.0	40.38	0.17
60°	5.27±0.50	36.7±11.1	0	7.0	50.19	0.14
70°	7.97±1.07*	55.5±18.7	51	7.0	75.90	0.09
80°	15.69±2.12*	109.4±36.8*	197	7.0	149.43	0.05

Values are mean±SD unless otherwise specified. This table documents the actual blood velocity and blood flow as functions of different insonation angles and the theoretical degree of potential error associated with different angles of insonation that will occur when normalizing FMD for shear.

*Data are significant ($P<0.05$) from 60°.

when the research focus is on sex differences, menses (days 1 to 7 of the menstrual cycle) offers the lowest attainable levels of both estrogen and progesterone in women and is, therefore, the optimum time for FMD studies.[4,43]

Previous Exercise/Rested State

A single bout of exercise has been documented to improve FMD in apparently healthy adults,[44] overweight men,[25] and postmenopausal women.[45] Therefore, it is important to be cognizant of the subject's physiological state; thus, it is recommended that subjects abstain from exercise for ≥12 hours before an FMD measurement.

Fasted State

There is considerable evidence describing the impact of the postprandial state on the FMD response. Indeed, the consumption of a single high-fat and high-carbohydrate meal has been shown to attenuate FMD in apparently healthy subjects[46,47] and in patients with type 2 diabetes mellitus,[48] in which oxidative stress and hyperglycemia, respectively, have been implicated. In contrast, it has been documented that the ingestion of a low-fat meal (ie, a corn flake cereal with skimmed milk) does not influence the FMD measurement.[46,47] Therefore, it is recommended that FMD assessments are performed under fasting conditions; however, if fasting is not possible, a standardized low-fat meal may be consumed before the FMD measurement.

Adequate Acclimatization

Because the goal of the FMD measurement is to compare the peak vasodilatory response with the baseline diameter, it is important that a true baseline be accurately assessed. Therefore, before an FMD test, it is recommended that subjects remain in the position in which the study will be performed (ie, supine, semisupine, or seated) for ≥20 minutes in a quiet, climate controlled room (22°C to 24°C) to control for orthostatic changes. In addition, a separate familiarization visit of the procedures is recommended to limit stress-induced sympathetic activity on the day of actual measurement.

Repeated Measurements

With respect to either having to repeat an FMD test or the nature of the study design (ie, repeated measures), it has been documented that multiple FMD tests can be validly performed if ≥30 minutes separates each measurement.[24] However, an important phenomenon to acknowledge is that FMD measurements exhibit diurnal variation,[49] and, thus, comparisons between and within subjects should be performed as consistently as possible with regard to the time of day.

In summary, appropriate subject preparation is essential to the successful ultrasonic assessment of FMD. Therefore, strict compliance in terms of limiting vitamin supplementation, cessation (or at least documentation) of medications, tobacco and caffeine use, phase of the menstrual cycle, previous exercise, and being fasted and rested before the FMD measurement is essential. Finally, if repeated measurements are necessary, an adequate amount of vessel recovery time should be allotted while also recognizing that there is diurnal variation in the FMD response.

Baseline Measurements

Once the investigator confirms that the subject has established a resting state (ie, several repeated and consistent measurements of blood pressure, blood velocity, and arterial diameter), baseline measurements should be performed. In addition, blood velocities provide an indication that a true resting state has been achieved and act as the starting point for the shear rate AUC calculations. The collection of accurate baseline diameters is essential for the valid FMD and shear rate calculations.

Baseline Arterial Diameter

The determination of baseline arterial diameters plays a pivotal role in the calculation and assessment of FMD. There is evidence to support similar diameters between resting and cuff occlusion conditions; however, recent data have emerged to indicate the impact of age[50] and cuff duration[51] on cuff occlusion–induced changes in arterial diameter. In addition, there is evidence to indicate a systemic difference in vascular endothelial function that appears to depend on the initial size of the artery.[52] In an effort to standardize the FMD methodology, it is recommended that ≥10 cardiac cycles be used in the calculation of baseline diameter. Because FMD is based on change in diameter, within reason, the actual borders (eg, intima-media or media-adventitia; Figure 2) that are used to determine the baseline and subsequent diameter are not as important as the need for consistency from baseline until maximal dilation. This having been said, it should be recognized that measuring from adventitia to adventitia rather than intima to intima will yield larger values for diameter (not actually representative of the vessel lumen), reducing the percentage of FMD change (because the baseline will be larger) and shear rate documented for the vessel being studied.

Baseline Blood Velocity

Blood velocity at rest plays an important role in the calculation of the shear response to cuff release, especially if using the AUC approach to assess shear rate.[53] With the growing recognition that shear stress is the predominant stimulus for the FMD response, accurate assessment of resting blood velocity is essential. Even at rest, in an unperturbed scenario, blood velocity over time can vary substantially (often because of heart rate variability); therefore, it is recommended that baseline blood velocity be averaged over at least a 10- to 20-second period.[54] In subjects who reveal a clear respiratory arrhythmia, this may need to be extended significantly, depending on respiratory rate, to reflect a true average basal blood velocity.

In addition to the clear need to optimize the insonation angle to accurately assess baseline blood velocities, placement and size of the sample volume (gate width) are of critical importance, especially when velocities are low. Figure 3 illustrates the impact of the Doppler sample gate size on the measurement of resting blood velocity and blood flow. In accordance with the hydraulic properties of Newtonian fluids, Figure 3 identifies ≈80% overestimation of volume flow when smaller sample volume sizes are used along the center of the vessel (Figure 3A, top) compared with a sample volume size spanning intima-to-intima (Figure 3C, bottom),
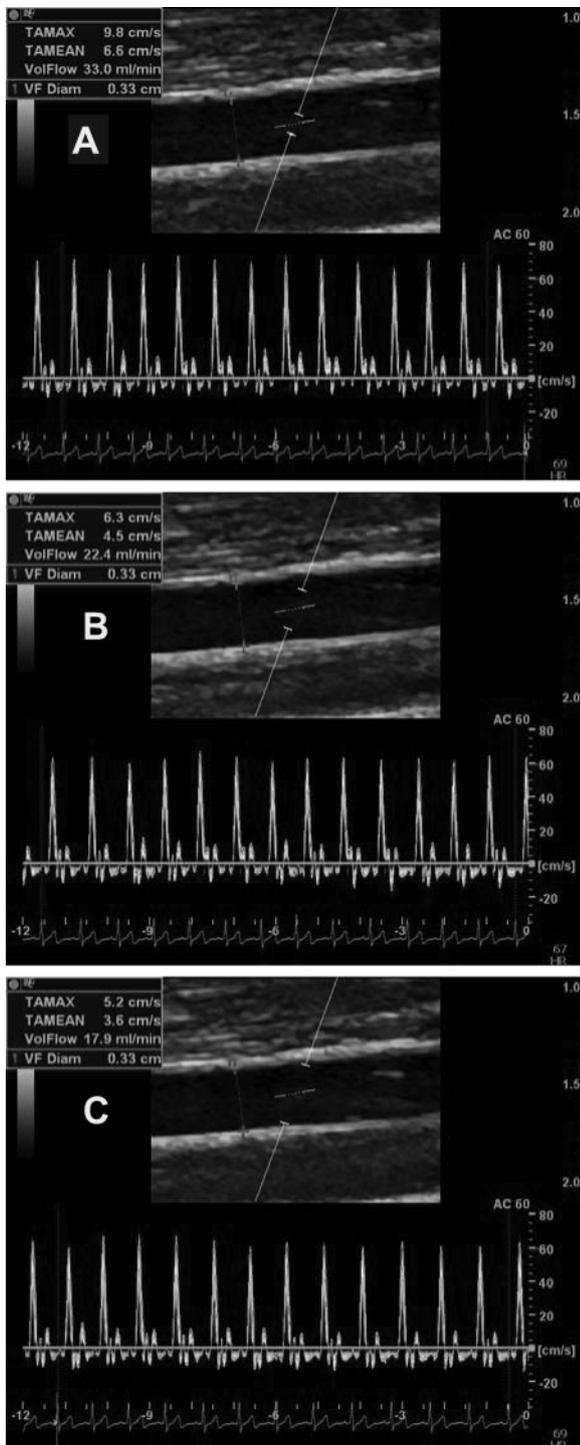
Figure 3. The determination of blood velocity and blood flow using different placements of the Doppler sample gate. A, Outer; B, middle; C, inner. Note the difference in velocity and blood flow among the different placements of the sample gate.

because of the laminar nature of blood flow and the reduction in blood velocity near the vessel wall. Thus, it is recommended that the sample volume be as wide as possible but without encompassing the vessel walls and allowing for a slight margin for error in case of movement (Figure 3B). Although the width of the gate will vary between laboratories, emphasis should be placed on maintaining consistency at the

very least between before and after cuff release within individual subjects and repeated measurements on the same subject.

In summary, the baseline measurements are extremely important components of an FMD study, because these assessments are built on after cuff release. Therefore, the accurate assessment of an average diameter and concurrent blood velocities, with appropriate sample volume, for ≥10 cardiac cycles is recommended before vascular occlusion (cuff inflation).

Vascular Occlusion
The initial stimulus for any FMD test relies on temporary vascular occlusion, which creates a region of ischemic tissue distal to the point of occlusion.[1] The metabolic byproducts of cellular respiration, in the absence of circulating blood, promote an increase in vascular conductance that allows a robust hyperemia on eradication of the "upstream" occlusion. This reactive hyperemia, and the associated shear stress experienced by the conduit vessels upstream from the area of occlusion, is the primary stimulus for FMD. Accordingly, both cuff position (proximal cuff=greater volume of tissue experiencing ischemia=greater hyperemia) and the duration of occlusion (longer occlusion=greater degree of ischemia=greater hyperemia) play integral roles in shear-mediated vasodilation.

The Cuff
The size of the cuff used for vascular occlusion should be appropriate for the area being occluded. Although a conventional hand-inflated cuff is adequate, it is both convenient and methodologically sound to almost instantaneously inflate and deflate the cuff, which can be achieved using a commercially available rapid (0.3-second) cuff inflator. It should be noted that a potential down fall of this rapid inflation approach is the "jolt" that can accompany both inflation and deflation, which can be avoided by supporting the arm in such a way that there is adequate space under the arm for the cuff to inflate and deflate.

Cuff Position
Although there is no consensus regarding the placement of the occlusion cuff relative to the site of measurement, there is growing support in favor of placement distal to the ultrasound probe, because this approach is thought to yield a predominantly endothelium-dependent vasodilation.[55] Positioning the cuff proximal to the imaging site elicits a greater peak hyperemic response and subsequent FMD[56] likely attributed to ischemia-induced hypoxia in the area being imaged. In addition, the decreased hyperemic decay observed after cuff deflation proximal to the site of measurement (Doppler probe) suggests that mechanisms in addition to NO-mediated vasodilation play a role in this scenario.[51,55]

Cuff Duration
Although it has been documented previously that a 10-minute cuff occlusion results in no greater maximal arterial dilation than a 5-minute occlusion,[4] for the sake of consistency and subject comfort it is recommended that a 5-minute suprasystolic cuffing period (200 to 250 mm Hg) be used for FMD tests. Although the link among vascular function in the

brachial artery, NO, and the duration of cuff occlusion is still a matter of some debate,[13,21] recent evidence supports the use of a 5-minute over a 10-minute occlusion, because sustained (>5-minute) occlusion may include more non-NO, ischemia-induced vasodilators.[57] Consistent with this, our laboratory has recently documented a 50% greater brachial artery vasodilation after 10 versus 5 minutes of occlusion, even after normalizing FMD for shear rate,[31] suggestive of nonendothelium-dependent, NO-mediated vasodilators playing a significant role after longer bouts of ischemia.

In summary, when performing an FMD test, the cuff should be the appropriate size for the limb being studied, positioned distal to the ultrasound probe, and inflated ≥25 to 50 mm Hg above systolic arterial pressure for 5 minutes to elicit a reactive hyperemic stimulus that is considered to be predominantly endothelium mediated and NO dependent.

Reactive Hyperemia (Postcuff Release) Measurements
The measurements after vascular occlusion (ie, postcuff release) are just as important, if not more so, than the baseline measurements. The following observations and recommendations apply to measurements during the postcuff release time period.

Temporal Kinetics of Arterial Diameters and Blood Velocities
As discussed above, it is recommended that Duplex mode on the ultrasound system be used. To ensure that no anomalies in arterial diameter occur during cuff occlusion and to capture the immediate hyperemic response, it is recommended that postcuff measurements be initiated ≥10 seconds before cuff release. Although the peak velocity occurs within the first 15 seconds, the peak vasodilation can be expected to occur 45 to 80 seconds after cuff release and may differ between populations.[58] Thus, it is also now recommended that the true peak diameter be determined on an individual basis (not simply the diameter in a given window of time) and the time to peak vasodilation be reported.

To capture the kinetics of reactive hyperemia-induced shear and subsequent vasodilation, it is recommended that blood velocity and diameter measurements be performed for ≥2 minutes after cuff release. Because the velocity profile after occlusion is characterized by a parabolic shape with exponential decay, it is generally agreed on that the integral of shear rate over time (ie, AUC) is the optimal method to quantify the accumulated shear that contributes to the FMD response.[53] AUC is conventionally calculated using the trapezoidal rule, according to the following equation:

$$(2) \quad \Sigma\{y_i[x_{(i+1)}-x_i]+(1/2)[y_{(i+1)}-y_i][x_{(i+1)}-x_i]\}$$

where x is time, y is shear, x_i is initial time point, and y_i is initial blood velocity.

Although Duplex mode is required to calculate the total shear rate (AUC), the previous recommendation to capture the peak hyperemic velocity during the first 15 seconds[4] before switching back to 2D imaging is still a feasible method, acknowledging the limitations of such an approach.

Calculation of Shear Rate
As described in the baseline velocities section and illustrated in Figure 3, the measurement of blood velocity with Doppler is influenced by the width of the sample volume and the placement of this gate within the vessel. This is the consequence of the parabolic velocity profile within unbranched sections of conduit vessels. This same concept has an impact on the calculation of shear rate, which is derived from the Poiseuilles law, dependent on Doppler sample volume size and placement: (1) large, centered sample volume: 8×mean blood velocity/internal diameter; or (2) small, centered sample volume: 4×mean blood velocity/internal diameter.

The difference in numerator (8 versus 4) of this calculation being explained by the failure to account for slower-moving red cells at the edge of vessel and, therefore, a bias toward an artificially elevated mean blood velocity as the sample volume becomes smaller but still located in the center of the vessel. On the basis of this information, it is recommended that the Doppler sample volume be kept wide and when shear rate is calculated the factor of 8 be used in the numerator of this equation.

In summary, it is recommended that both diameter and velocity data be acquired for ≥10 seconds before cuff release and continue these data collection for ≥2 minutes postrelease. This method will not only allow for the documentation of the true peak diameter but it will also allow the quantitative analysis of shear AUC, the stimulus thought to be predominantly responsible for the FMD response. In addition, documenting the time to peak vasodilation may more appropriately assess endothelial function when making comparisons among different groups and/or clinical populations.

FMD Analyses
Recently, it has become apparent that the measurement of FMD may not be as simple as assessing vessel diameter both before and after cuff release and reporting a percentage of increase in vessel caliber. Indeed, over the past 2 decades, both the methodology and analysis of FMD have received significant attention, evolving into what are recommended today. For example, it is now acknowledged that, when using the traditional percentage of change calculation, the initial baseline diameter has the potential to introduce mathematical bias into the FMD assessment, with smaller vessels appearing more reactive and vice versa.[7] Therefore, it is now recommended that, in addition to FMD expressed as a percentage, researchers document baseline diameters, absolute change in diameter, and shear rate (AUC).

Edge Detection Software
The use of edge detection software for offline analysis is recommended for the measurement of baseline and postcuff release diameters. Using this approach facilitates more objective and accurate diameter measurements and also permits synchronization with the ultrasound system and ECG to allow sequential end-diastolic images to be stored, avoiding artifacts attributed to pulse-related changes in vessel diameter. Currently, the most commonly used, commercially available edge detection software is that developed by Medical Imaging Applications LLC. This edge detection software has been independently validated[59] and is now commonly found in the literature.[25,31,35,60–62]

If edge detection software is unavailable, it is recommended that data (diameter and velocity) be collected every 4

Table 2. Absolute Difference and Variability Between Manual and Software Evaluations of FMD Determinants

Subject	Baseline Diameter, cm				Peak Diameter, cm				FMD, %			
	Manual	Software	Difference, cm	CV, %	Manual	Software	Difference, cm	CV, %	Manual	Software	Difference, cm	CV, %
1	0.42	0.4000	0.015	2.6	0.44	0.4200	0.020	3.3	6.02	5.00	1.02	13.1
2	0.43	0.4497	−0.022	3.6	0.45	0.4689	−0.019	2.9	5.26	4.27	0.99	14.7
3	0.40	0.3951	0.002	0.4	0.41	0.4027	0.007	1.3	3.14	1.92	1.22	34.1
4	0.34	0.3475	−0.007	1.5	0.35	0.3595	−0.010	1.9	2.94	3.45	−0.51	11.3
5	0.31	0.3200	−0.010	2.2	0.35	0.3511	−0.001	0.2	12.90	11.42	1.48	8.6
6	0.30	0.3029	−0.003	0.7	0.32	0.3244	−0.004	1.0	6.67	8.13	−1.47	14.0
7	0.33	0.3200	0.010	2.2	0.37	0.3600	0.010	1.9	12.12	12.50	−0.38	2.2
8	0.24	0.2400	0.000	0.0	0.27	0.2900	−0.020	5.1	12.50	20.83	−8.33	35.4
9	0.29	0.2833	0.009	2.3	0.31	0.3100	0.000	0.0	5.98	9.41	−3.43	31.5
10	0.33	0.3204	0.006	1.4	0.36	0.3483	0.012	2.3	10.20	8.71	1.50	11.2
11	0.29	0.2733	0.017	4.2	0.31	0.2900	0.020	4.7	6.90	7.41	−0.51	5.1
12	0.25	0.2500	0.003	0.9	0.27	0.2600	0.010	2.7	6.58	5.41	1.17	13.8
13	0.38	0.3900	−0.010	1.8	0.39	0.3974	−0.007	1.3	2.63	2.03	0.60	18.3
14	0.38	0.3800	0.000	0.0	0.38	0.3807	−0.001	0.1	0.00	0.00	0.00	0.0
15	0.30	0.3000	0.000	0.0	0.31	0.3100	0.000	0.0	3.33	3.33	0.00	0.0
Mean	0.33	0.3315	0.001	1.6	0.35	0.3515	0.001	1.9	6.48	6.92	−0.4	15.2

Note that there is a greater accuracy of diameter measurements and the subsequent determination of FMD using software analysis.

seconds for the first 20 seconds after cuff release, followed by every 10 seconds for the remaining 2-minute data collection period. Table 2 illustrates the variability and absolute differences in baseline diameter, peak diameter, and calculated FMD between careful manual (calipers on the ultrasound) and edge detection software evaluation in a range of subjects. Although strong correlations and low variability exist for baseline diameter ($r=0.98$; coefficient of variation [CV]: 1.9%), peak diameter ($r=0.98$; CV: 1.9%), and calculated FMD ($r=0.89$; CV: 15.2%), it does appear that using edge detection software not only provides a more robust and sensitive assessment of FMD, it also removes any subjective error component from the data analysis.

Calculation of FMD
The calculation of FMD as a percentage change uses the peak diameter in response to reactive hyperemia in relation to the baseline diameter and is calculated using the following equation:

$$(3) \quad FMD(\%)=\frac{\text{peak diameter}-\text{baseline diameter}}{\text{baseline diameter}}$$

and when multiplied by 100, FMD is expressed as a percentage of change in the vessel caliber.

It is recommended that the average diameter assessed during end diastole (identified by the R wave on the ECG) over ≥ 10 cardiac cycles be used to represent the baseline diameter. With the introduction of the edge detection software, some debate has developed as to the optimum time resolution necessary to accurately determine peak diameter.[58] The differences in FMD and determinants using 3-, 5-, and 10-second data smoothing averages are presented in Table 3. Indeed, if diameters are averaged (data smoothing) over some period of time (ie, 10 seconds), the "exact" peak will lose resolution. However, data smoothing with a time period that

is too short (ie, 3 seconds) inevitably increases the noise of the measurement and may result in identification of an aberrant value for peak diameter. Accordingly, it is recommended that the peak diameter be determined over as short a period of time as possible (ie, 5 seconds) but never relying on peak diameter data that are obtained from the average of <3 measurements (ie, 3 cardiac cycles). The data-smoothing time period should be reported and blood velocities should be analyzed during the same time frame as the diameters (ie, Duplex mode).

Normalization of FMD (FMD/Shear)
Because FMD is thought to be evoked by shear stress and, thus, proportional to reactive hyperemia, consideration of the heterogeneity of blood flow responses across subjects deserves greater attention than it received at the outset of such endothelial function measurements.[4] Indeed, it has been suggested recently that FMD should be normalized by dividing the percentage of FMD by shear rate (AUC).[53] Although this mathematical correction for shear stimulus is theoretically simple, experimental evidence supporting normalization is equivocal. In the study of vascular aging, it has been

Table 3. Difference in FMD and Determinants Using 3-, 5-, and 10-Second Data Smoothing Averages

Variable	3 Seconds	5 Seconds	10 Seconds
Baseline diameter	3.25±0.15	3.25±0.15	3.25±0.15
Peak diameter	3.45±0.15	3.43±0.15*	3.42±0.15*
FMD, %	6.7±0.9	6.0±0.8	5.6±0.7*
Time to peak, seconds	44±6	43±4	44±6
No. of frames	2.4±0.3	4.0±0.4*	7.7±0.7*†

Data are presented as mean±SEM.
*Data are significant ($P<0.05$) from 3 seconds.
†Data are significant ($P<0.05$) from 5 seconds.

reported that healthy older adults have a preserved endothelial function when FMD is normalized for their reduced postcuff release shear rates.[32] In addition, Padilla et al[62] demonstrated that normalizing FMD for shear rate (AUC) eliminates the influence of differing shear profiles created by varying periods of cuff ischemia. Additional data to support normalization have been indicated by the removal of limb-specific differences in FMD when shear rate was used to normalize the FMD response.[63] In contrast, a recent study has concluded that normalizing FMD for shear is age dependent and only appropriate when investigating young adults.[64] Therefore, the use of Duplex ultrasound and the subsequent ability to assess FMD, measure reactive hyperemia, and calculate shear AUC are emerging as important components of FMD measurements made with ultrasound Doppler. In addition, reactive hyperemia alone has been documented to have high clinical prognostic value.[65]

In statistical terms, the appropriate method by which to take differing shear rates into account during an FMD study is complex. To legitimately apply such a correction factor to a variable, the relationship between the 2 variables must satisfy 3 assumptions: (1) a significant correlation; (2) the y intercept of this relationship must be 0; and (3) the data should be normally distributed. From our own experience and that of others (Figure 4 and References 53 and 64), the relationship between vessel dilation and shear rate often violates ≥1, if not all 3, of these assumptions, leaving serious doubt as to whether a simple mathematical normalization should be categorically performed. If, as has been suggested, there is simply a modest correlation between FMD and shear and the shear stimulus differs between independent variables, the proper method of taking into account the covariance of shear rate with FMD may be through the ANCOVA,[66] although, as of yet, this method is also not completely accepted.

Figure 4 illustrates the relationships between FMD and peak shear, shear AUC up to the time of peak dilation, and total shear AUC (2 minutes) in both young and old subjects. Although all of the relationships for the young population appear to be very strong, it is important to note that only the shear AUC until peak vasodilation (Figure 4, plot B) yields the strongest relationship in both age groups. These data are in agreement with previous work[53] and provide further evidence that shear rate (AUC) until the time of peak dilation may be the most appropriate method of quantifying shear forces.

In summary, edge detection software has been independently validated and is recommended for the measurement of arterial diameter. To standardize the FMD technique, the recommended procedure for obtaining diameters is through continuous digital data recording and offline analysis using edge detection software. To identify and calculate the FMD response, the true peak diameter is obtained and expressed as an increase in vasodilation above baseline values. Although normalization of FMD for shear has been embraced by many researchers, uncertainty currently exists as to how to properly normalize FMD. It is currently recommended that FMD normalized for shear rate (AUC) be calculated and reported but that the raw shear (AUC up to peak vasodi-
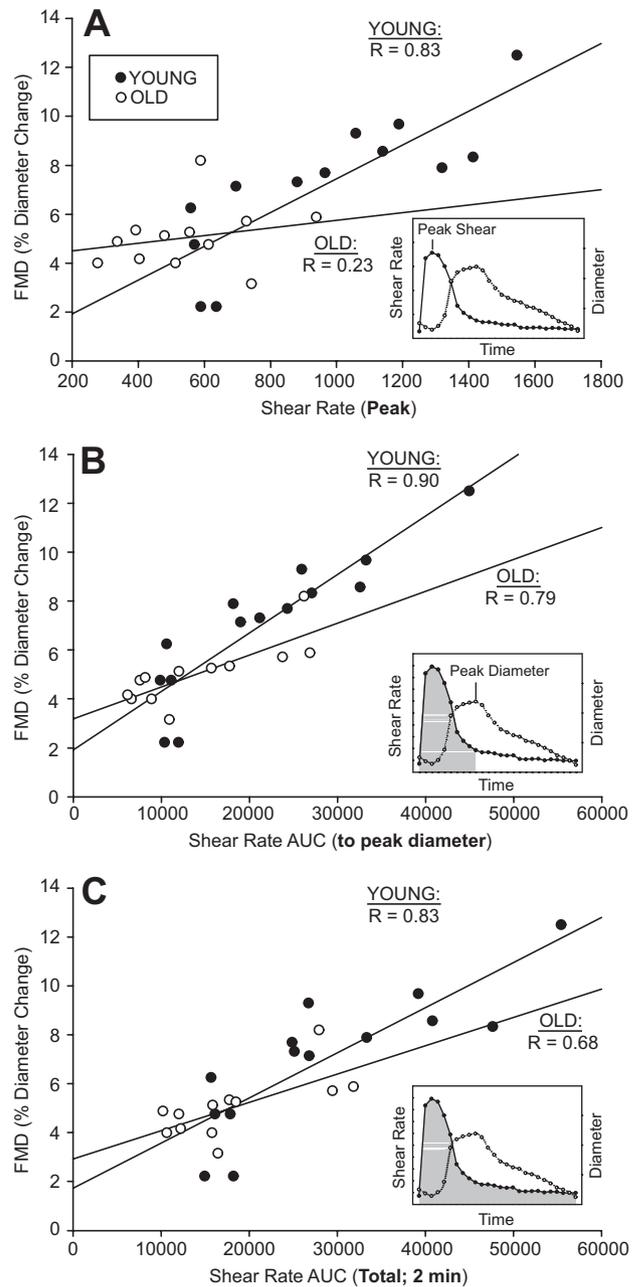

Figure 4. The relationships between FMD and different assessments of shear rate to be considered when normalizing FMD. A, FMD vs peak shear; B, FMD vs shear AUC until peak diameter; C, FMD vs total shear AUC for the entire 2 minutes. Inlays for each panel illustrate the corresponding shear rate (shaded) used in the analysis.

lation) and FMD data also be readily available to allow for alternative analyses or interpretations. In addition, the time it takes to obtain peak vasodilation may be an important indicator of stimulus sensitivity that should be incorporated into an evaluation of endothelial function by an FMD test.

Conclusion
The measurement of FMD is often mistaken as a simple noninvasive method of assessing vascular endothelial function that anyone with access to an ultrasound Doppler can

perform.[13] However, the appropriate ultrasound technology, subject preparation, and knowledge of the method are required to perform an accurate assessment of endothelial function using the FMD technique. The recommendations proposed in this comprehensive tutorial represent the most recent advancements in the ultrasonic measurement of FMD and are presented in an attempt to standardize this measurement across research sites and to subsequently facilitate the use of FMD as a clinically relevant research tool.

Disclosures

None.

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