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Assessment of Endothelial Function Using Ultrasound

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1. Introduction

The pathological complications of atherosclerosis, namely heart attacks and strokes, remain the leading cause of mortality in the Western world (Lloyd-Jones & Adams *et al.* 2010). Preceding atherosclerosis is endothelial dysfunction (Ross 1993; Cohn 1999; Quyyumi 2003). The endothelium comprises a continuous monolayer of cells which separate the vascular wall from the circulation (Lerman & Zeiher 2005). Disruption of this essential monolayer is thought to occur early in the pathogenesis of cardiovascular disease (CVD). There is, therefore, interest in the application of non-invasive clinical tools to assess the function and health of this essential monolayer.

The flow-mediated dilation (FMD) test is the standard tool used to assess endothelial function (Celermajer & Sorensen *et al.* 1992). Reduced FMD is an early marker of atherosclerosis (Celermajer & Sorensen *et al.* 1992), has been noted for its capacity to predict future CVD events (Schroeder & Enderle *et al.* 1999; Neunteufl & Heher *et al.* 2000; Heitzer & Schlinzig *et al.* 2001; Murakami & Arai 2001; Yoshida & Kawano *et al.* 2006; Inaba & Chen *et al.* 2010), and an impaired vascular response has also been demonstrated in children as young as 7 years old with familial hypercholesterolemia (Sorensen & Celermajer *et al.* 1994). This review discusses the measurement of endothelial function, with a focus on the FMD technique.

2. The vascular endothelium

From the lumen to the outer wall all arteries are composed of an intima, media, and adventitia (see Fig. 1). The adventitia is the outer most layer, and is mainly composed of connective tissue that maintains vessel shape and limits distention. The media is comprised mainly of vascular smooth muscle cells that regulate blood flow by vasoconstriction or vasodilation. The intima is the inner most lining of the vessel, and consists of the endothelium and underlying connective tissue.

Vascular endothelial cells essentially have the same characteristics as all the cells of the human body: cytoplasm and organelles surrounding a nucleus and contained by the cellular membrane. Endothelial cells form a continuous flat mono-layer that cover the vascular lumina throughout the arterial tree. The endothelium is mechanically and metabolically

strategically located, separating the vascular wall from the circulation and the blood components (Lerman & Zeiher 2005).

The vascular endothelium utilizes autocrine, paracrine, and classical endocrine signaling to promote vascular homeostasis (Luscher & Barton 1997). These cells are capable of producing a variety of agonistic and antagonistic molecules, including vasodilators and vasoconstrictors, pro-coagulants and anti-coagulants, inflammatory and anti-inflammatory, fibrinolytics and anti-fibrinolytics, oxidizing and anti-oxidizing, and many others (Luscher & Barton 1997).

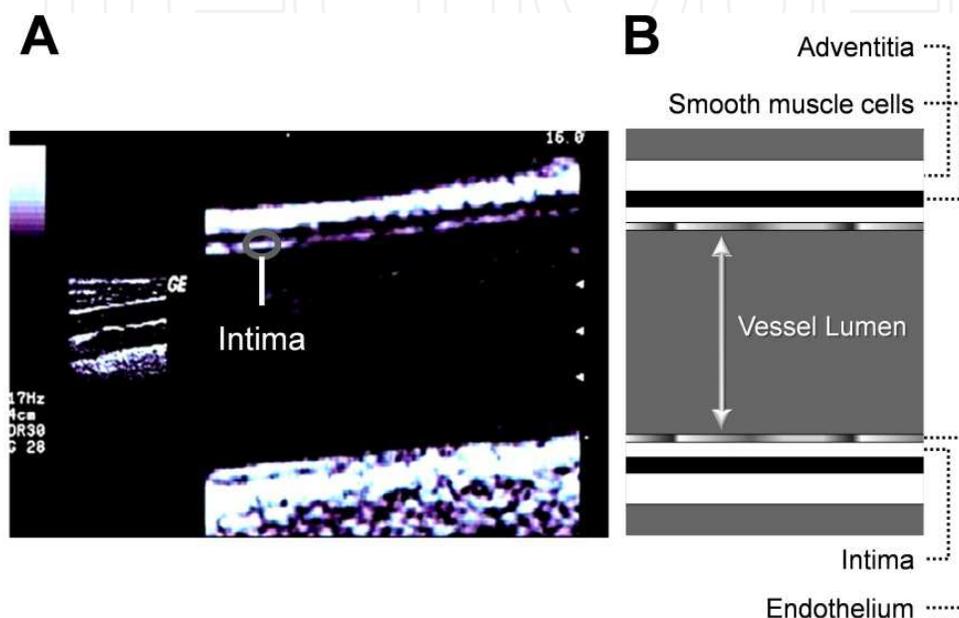


Fig. 1. *Anatomy of the arterial wall.* (A) A conduit artery imaged in the longitudinal plane using ultrasound. (B) The layers comprising the wall of an artery. Endothelial cells form a continuous layer lining the intima throughout the arterial tree.

2.1 Endothelial dysfunction and atherosclerosis

Upsetting the delicate balance of functions performed by the endothelium initiates a number of events that promote atherosclerosis, the precursor to CVD. Although atherosclerosis is commonly described as the presence of plaques that obstruct the lumen of the conduit arteries, endothelial dysfunction precedes plaque formation (Gibbons & Dzau 1994; Ross 1999; Nissen & Yock 2001). Reduced endothelial responses can be observed early in the course of atherogenesis, preceding angiographic or ultrasonic evidence of atherosclerotic plaque (Luscher & Barton 1997).

Disruption of the functional integrity of the vascular endothelium plays an integral role in all stages of atherogenesis, ranging from lesion initiation to plaque rupture. Endothelial dysfunction leads to increased permeability to lipoproteins, foam cell formation, T-cell activation, and smooth muscle migration into the arterial wall (Ross 1999). The first step in the formation of the plaque occurs when the inflammatory response is incited and fatty streaks appear. If these conditions persist, fatty streaks progress and the plaques become vulnerable to rupture.

Parameter	Recommendations
<i>Subject preparation</i>	Fast overnight prior to testing, and avoid exercise during the preceding 24 hrs. Refrain from taking drugs with known vascular effects. Rest supine for 20 mins in a quiet, temperature controlled room at 21 °C. Test conducted with subjects in the supine position. Artery segment of interest must remain at or below heart level. Women should be tested during the early follicular phase of the ovarian cycle (i.e., day 7-14 of the ovarian cycle). For successive tests, subjects should report at the same time of day to reduce error associated with circadian variation.
<i>Probe selection</i>	A higher frequency probe (12MHz) should be used for superficial arteries (e.g., brachial, radial or posterior tibialis). A lower frequency probe (7.5MHz) should be used for deeper arteries (e.g., common femoral). The same transducer should be used for all subjects in a given study.
<i>Probe placement</i>	Mark anatomical placement for studies with repeated measurements. Use a probe holding device to maintain image focus.
<i>Ultrasound Settings</i>	Standardize ultrasound global (acoustic output, gain, dynamic range, gamma, rejection) and probe-dependent (zoom factor, edge enhancement, frame averaging, target frame rate) settings.
<i>Artery</i>	Artery selection should be made based on the population of interest, e.g., lower limb arteries should be measured in patients with SCI.
<i>Diameters (general)</i>	Extend across the entire imaging plane to minimize skewing prior to focusing. Use automated or semi-automated image analysis software. Use mean or end-diastolic diameters.
<i>Baseline diameters</i>	Collect prior to cuff inflation. Subject should hold breath during measurement. Collect and average 3 * 10 sec measurements.
<i>Peak diameters</i>	Capture diameters continuously to ensure true peak diameter.
<i>Blood velocity</i>	The beam-vessel angle must be $\leq 60^\circ$. Measure continuously. Time-averaged maximum velocities are more accurate and reproducible than time-averaged mean velocities.
<i>Shear Stimulus</i>	Shear rate is a suitable substitute for shear stress. Diameters and velocities must be captured continuously to estimate shear. Shear rates should be presented as an integral, we recommend 40 secs post-ischemia. Attention should be paid to secondary flow phenomena, e.g., turbulence and velocity acceleration.
<i>Analysis</i>	Present FMD in absolute (mm) and relative (%) terms. The shear rate stimuli should be presented for each research setting. Do not normalize FMD to shear rate as ratio or using ANCOVA. HLM can be used to statistically account for shear rate in the evaluation of FMD.

Table 1. Recommendations for FMD Testing

2.2 Stimuli regulating endothelial function

The haemodynamic conditions inside blood vessels lead to the development of superficial stress near the vessel walls which can be divided into two categories: 1) circumferential stress due to pulse pressure variation inside the vessel, and 2) shear stress (Nerem 1992; Papaioannou & Stefanadis 2005; Papaioannou & Karatzis *et al.* 2006). Circumferential stress acts perpendicular to the vessel wall, whereas shear stress acts at a tangent to the wall to create a frictional force at the surface of the endothelium. Circumferential stress applies stress to all layers of the vessel wall (intima, media and adventia), while shear stress is applied principally at the endothelial surface. Shear stress is considered to be the primary stimulus regulating endothelial cell function.

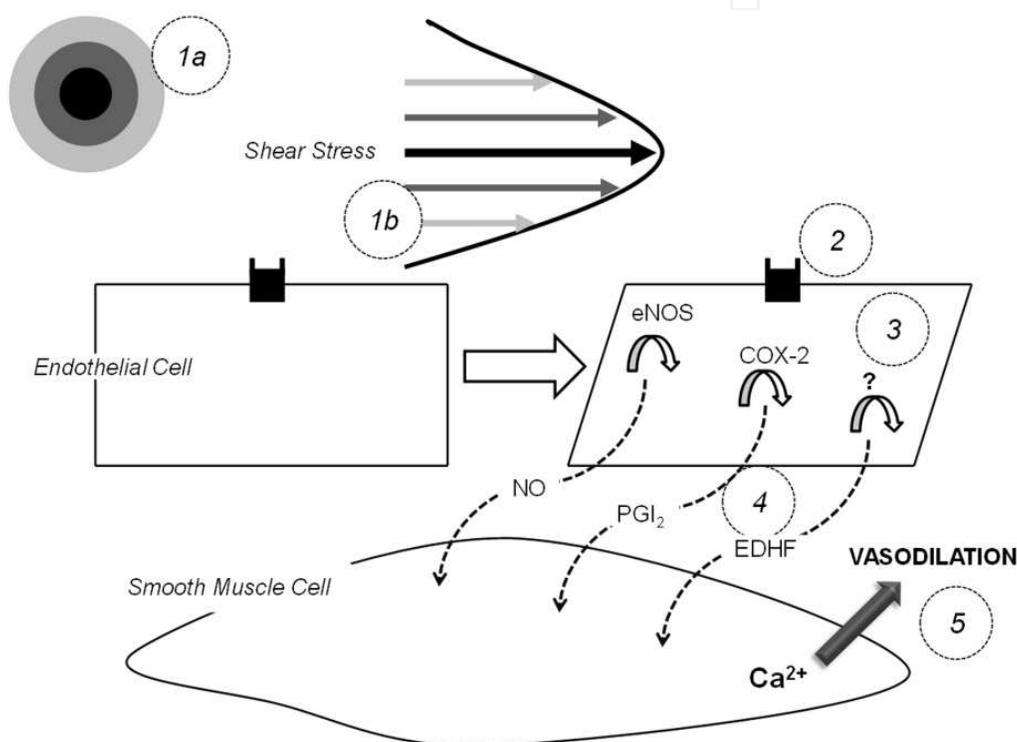


Fig. 2. *Endothelium-dependent dilation.* (1) Blood flowing through an artery creates a shearing stress at the endothelial surface. A composite of superimposed concentric circles is shown in 1a (i.e., transverse plane) to correspond with the gradient of increasing RBC velocity from the periphery to the center of the lumen. RBC velocity is represented as a parabola (i.e., longitudinal plane) in 1b using the same color coding as in 1a. The magnitude of the parabola (left to right) corresponds with the gradient of increasing RBC velocity from the periphery to the center of the lumen. (2) Shear stress-induced deformation of the endothelial cells is detected by mechanoreceptors on the cell membrane. (3) In response to mechanotransduced shear stress, a signaling cascade results in the production of NO, PGI₂ and EDHF. (4) The vasodilators diffuse across the interstitial space and enter the vascular smooth muscle cells. (5) A signaling cascade is initiated which lowers Ca²⁺ concentration and results in smooth muscle cell relaxation (i.e., vasodilation). Ca²⁺ = calcium; eNOS = endothelial NO synthase; COX-2 = cyclooxygenase; EDHF = endothelial-derived hyperpolarizing factor; NO = nitric oxide; PGI₂ = prostaglandins; RBC = red blood cell.

Shear stress is primarily related to movement of red blood cells close to the endothelial layer (represented by bottom and top-most arrows in Fig. 2.1b). As fluid particles “travel” parallel to the vessel wall, their average velocity increases from a minimum at the wall to a maximum value at some distance from the wall, resulting in a gradient of velocities that form concentric circles in the lumen of the vessel (Fig. 2.1a). This shearing stress therefore acts at a tangent to the wall to create a frictional force at the surface of the endothelium. Although shear stress has a very small magnitude in comparison to circumferential stress, the endothelial cells are equipped with numerous mechanosensors to detect this stress (Olesen & Clapham *et al.* 1988; Davies 1995; Barakat & Leaver *et al.* 1999; Shyy & Chien 2002; Fleming & Busse 2003; Labrador & Chen *et al.* 2003). To maintain physiological levels of vessel wall shear stress, vascular tissues respond to changes in shear stress with acute adjustments in vascular tone (through vasodilation) (Langille & O'Donnell 1986). Vasodilation reflects alterations in the rate of production of endothelial-derived mediators, including nitric oxide (NO), prostacyclin (PGI₂) and endothelium derived hyperpolarizing factor (EHRF), which act locally to modulate vascular smooth muscle tone (see Fig. 2).

3. Flow-mediated dilation testing

In 1970, Rodbard (Rodbard 1970) proposed that the endothelium may sense and respond to shear stress generated by flowing blood. In 1980, Furchgott and Zawadzki (Furchgott & Zawadzki 1980) discovered that agonist-mediated vasodilation requires participation by the endothelium. The dependence of FMD on an intact endothelium was subsequently shown to occur in large-conduit arteries as well as in resistance-sized vessels (Rubanyi & Romero *et al.* 1986). More recent studies have demonstrated that vasodilation is directly proportional to increases in shear stress (Koller & Sun *et al.* 1993; Moncada & Higgs 1993).

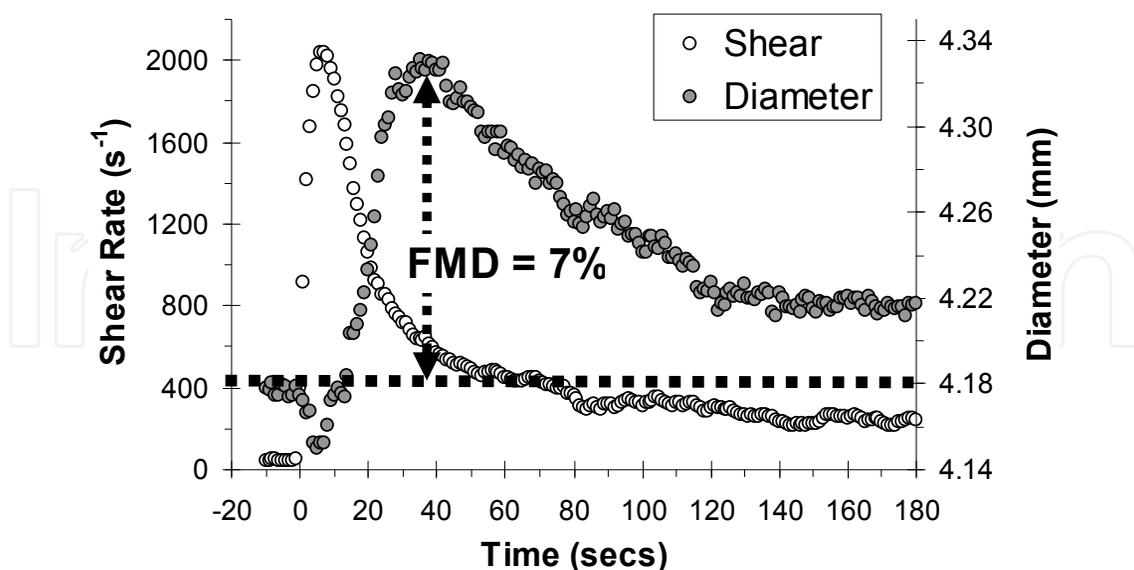


Fig. 3. Shear rate and diameter responses to 5 minutes ischemia. The horizontal line represents resting diameter. Flow-mediated dilation (FMD) is typically represented as the peak percentage increase in diameter above rest. Note that the peak diameter occurs ~40 sec whereas the bulk of the hyperemic (shear) response occurs within the initial 20 sec.

Endothelium-dependent agonists, such as acetylcholine, can be used to induce a dilatory response (Furchgott & Zawadzki 1980). However, such practice is invasive and often unpractical, especially for use within clinical settings. Alternatively, the FMD test is a *non-invasive* method (Fig. 3). Typically, a pneumatic tourniquet will be placed around the forearm approximately 5cm below the olecranon process and inflated to a super-systolic blood pressure for 5 minutes. Rapid deflation of the tourniquet instigates increased blood flow (reactive hyperemia) to the oxygen starved forearm muscles, with a subsequent increase in flow through the upstream brachial artery. The flow-induced increase in shear stress results in vasodilation. FMD is typically expressed as the percentage increase in the artery diameter above baseline. Table 1 provides a list of recommendations to consider when conducting this test.

4. Ultrasound

Arndt (Arndt & Klauske *et al.* 1968), in 1968, was the first to apply ultrasound to [carotid] arterial measurements. Since then, the advancement of ultrasound technology has had a profound impact on the capacity of researchers and clinicians alike to non-invasively assess endothelial function and health. Most commercial ultrasound machines now provide duplex Doppler functionality; that is, they can simultaneously image and measure blood velocity in conduit arteries in real-time. Duplex Doppler functionality offers immense potential for tracking vascular mechanical and functional changes.

4.1 Arterial diameter measurements

Conventionally, two-dimensional brightness mode (B-mode) is used to visualize, in real-time, the ultrasound echo amplitude distribution in a tomographic plane. The arteries of interest, except for the aorta, are typically within a depth range of 30 mm; a high carrier frequency (typically 7–13 MHz) is used to provide detailed images of peripheral arteries, in both longitudinal and cross-sectional views (Hoeks & Brands *et al.* 1999). Ultrasound wave reflections will only have a prominent amplitude if they originate from acoustic interfaces with a substantial change in acoustic impedance and, are oriented perpendicular (i.e., at a 90 degree angle) to the ultrasound beam direction. Therefore, in the cross-sectional view the lateral segments of the artery wall are blurred, with relatively low amplitude for the anterior and posterior lumen-wall transitions. In the longitudinal view, both walls will show up distinctly over a certain range, provided that the arterial segment considered is straight and without branches (Fig. 1). The transition of the inner layer of the wall, the intima to the lumen, induces a weak signal while the outer layer, the adventitia, results in reflections with high amplitude. The layer in between, the media, has a relatively low reflectivity and appears as a hypo-echoic band in images obtained with ultrasound systems with sufficient resolution.

A number of laboratories, using commercial or custom edge-detection software, are now able to make semi-automated diameter measurements (Woodman & Playford *et al.* 2001; Craiem & Chironi *et al.* 2007; Peretz & Leotta *et al.* 2007; Padilla & Johnson *et al.* 2008; Pyke & Jazuli 2011; Thijssen & Tinken *et al.* 2011). The authors of this chapter, using custom edge-detection software, are able to make thirty diameter measurements per second. A video capture device is used to make recordings at a rate of 30 frames / second. These video files are broken down and converted into JPEG (Joint Photographic Experts Group) images, which provides

comparable accuracy for ultrasound image measurements compared to the DICOM (Digital Image and Communications in Medicine) standard (Hangiandreou & James *et al.* 2002). The images are analyzed offline using semi-automated edge-detection software (Fig. 4) custom written to interface with National Instruments LabVIEW software (National Instruments, Austin, TX, USA) (Sabatier & Stoner *et al.* 2006; Stoner & Sabatier *et al.* 2006). Custom-written Visual Basic Code is used to fit peaks and troughs to diameter waveforms in order to calculate diastolic, systolic, and mean diameters. The authors use mean diameters for analysis. Traditionally, end-diastolic diameters were used to calculate FMD, owing to: 1) FMD measurements that incorporate non-end-diastolic diameters may introduce measurement errors due to fixed vessel structural issues, and 2) prior to the advent of automated image analysis software, mean diameter measurements were beyond the technical capabilities of most research units. A recent study indicates that calculating FMD based on mean diameters yields comparable results to calculations based on end-diastolic diameters (Kizhakekuttu & Guterman *et al.* 2010). The within-session SEM_{3,1} for the described set-up is 0.046 mm; between-day coefficients of variation are 2.4-2.7% (Stoner & Sabatier *et al.* 2004).

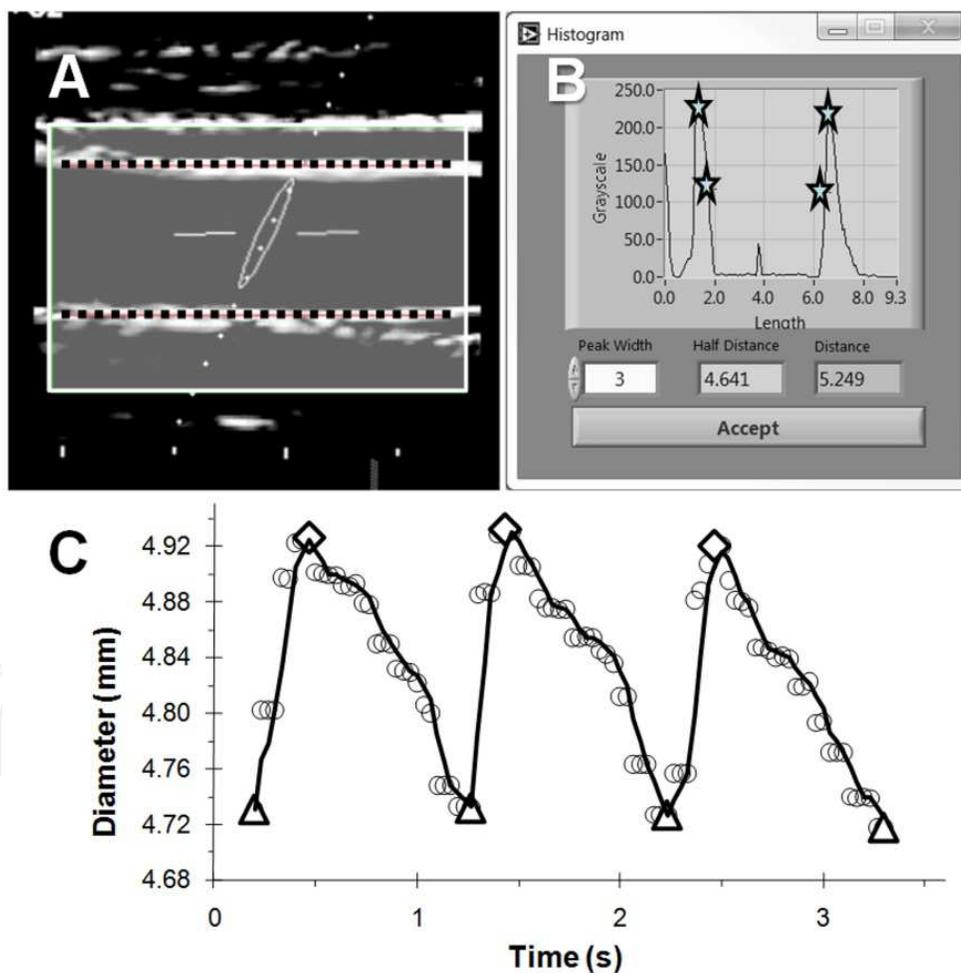


Fig. 4. *Semi-automated diameter analysis.* (A) B-mode image of the brachial artery with a region of interest (ROI) denoted by a selection box. The histogram (B) corresponds with the average pixel brightness of rows in the ROI in (A). The peaks (stars) correspond with the vessel walls. (C) Diameter waveforms from three cardiac cycles. Triangles represent diastole and diamonds represent systole.”)

4.1.1 Measurement protocol

When imaging a vessel care should be taken to ensure that the vessel clearly extends across the entire [un-zoomed] plane to minimize likelihood of skewing the vessel walls. The ultrasound transducer should then be adjusted until the vessel walls appear thickest. Ultrasound global (acoustic output, gain, dynamic range, gamma, rejection) and probe-dependent (zoom factor, edge enhancement, frame averaging, target frame rate) settings should be standardized, especially for a given study. Alterations to probe selection and optimization settings - particularly probe selection - can have a significant impact on measurement precision (Stoner, in press). Figure 5 shows two diameter waveforms, both measured on the same subject within 10 minutes, albeit with different probes (11MHz and 6.6MHz); despite all other global and probe-dependent settings being equal, the 6.6mhz resulted in bias for smaller diameters. To ensure image focus is maintained and that diameter waveforms are stable, the ultrasound probe needs to be fixed in place using a probe holding device. The stability of diameter waveforms is also affected by rhythmic breathing patterns; to ensure optimal quality of diameter waveforms, the subject should ideally hold their breath during image acquisition.

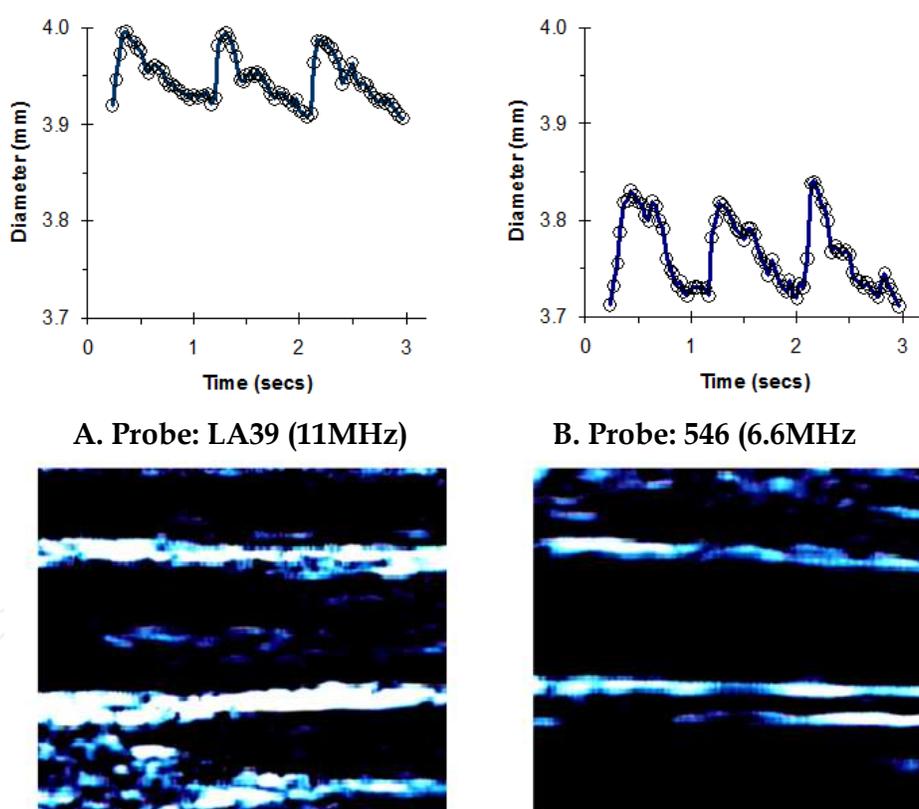


Fig. 5. Brachial artery diameter waveforms using a GE 11MHz (A) and 6.6MHz (B) probe. Measurements were taken from the same subjects within 10 minutes of one another. Note the bias towards smaller diameters using the 6.6Mhz probe.

4.1.2 Probe selection

Selection of the appropriate ultrasound probe is dependent on the vessel being imaged. The higher the probe frequency the greater the axial resolution, but this comes at the cost of

tissue penetration (Roelandt & van Dorp *et al.* 1976; Lieu 2010). The operator should use the highest frequency that has adequate tissue penetration to clearly resolve the structure of interest. For superficial arteries, e.g., brachial, radial and posterior tibialis, a 12Mhz probe will allow adequate penetration and will provide optimal axial resolution. When imaging deeper arteries, e.g., common femoral, a 12MHz probe will not provide sufficient penetration, and a lower frequency probe (e.g., 7.5MHz) is recommended. However, the use of a lower frequency probe will yield a lower axial resolution, and will limit the capacity to discern small changes in vessel diameter. The same transducer should be used across subjects for a given study to maximize statistical power (see Fig. 5).

4.2 Blood velocity measurements

Ultrasound assessments of blood velocity have been favorably compared to magnetic resonance imaging, which is capable of higher resolution but is much more costly (Nesbitt & Schmidt-Trucksass *et al.* 2000). With ultrasound, blood velocity is calculated by measuring the Doppler shift, which results from a change in the frequency of a wave due to the motion of the wave source or receiver, or in the case of a reflected wave, motion of the reflector. The Doppler shift is dependent on the insonating frequency, the velocity of moving blood, and the angle between the sound beam and direction of moving blood, as expressed in the Doppler equation:

$$Df = \frac{2 \cdot f \cdot v \cdot \cos q}{c} \quad (1)$$

where: Df is the Doppler shift frequency (the difference between transmitted and received frequencies), f is the transmitted frequency, v is the blood velocity, q is the angle between the sound beam and the direction of moving blood, and c is the speed of sound. The equation can be rearranged to solve for blood velocity, and this is the value calculated by the ultrasound machine:

$$v = \frac{Df \cdot c}{2 \cdot f \cdot \cos q} \quad (2)$$

Since red blood cells travel at different speeds, even for a small measuring volume, there will be a range of blood velocities for a given unit of time. Per cardiac cycle, Doppler ultrasound systems measure minimum, maximum, and mean blood velocities. The maximum velocities represent the fastest moving blood cells flowing through the center of the vessel, whereas mean-velocities represent the average speed of blood cells from across the vessel. Mean velocities tend to be limited by incomplete sampling of Doppler shifts across the full width of the artery (Thrush & Hartshorne 2004). Time-averaged maximum velocities are more accurate and reproducible, even though they may lead to overestimations of blood flow by approximately 40% (Olive & Slade *et al.* 2003).

4.2.1 Measurement protocol

Most commercial ultrasound units come equipped with software to automatically calculate blood velocities. These automated calculations are typically limited to each heart beat. When making simultaneous diameter and blood velocity measurements, a compromise has to be

made. An optimal B-Mode image is obtained when the ultrasound probe is perpendicular (90 degrees) to the imaged vessel, whereas an optimal velocity signal is obtained with a beam-vessel angle $\leq 60^\circ$. Error associated with incorrect estimation of insonation angle increases exponentially with angles $\geq 60^\circ$ (Thrush & Hartshorne 2004). For further discussion on this topic see Thijssen et al. 2011 (Thijssen & Black *et al.* 2011).

4.3 Measurement location

For two decades brachial artery FMD has been widely used as a global endothelial health index (Celermajer & Sorensen *et al.* 1992). However, the brachial artery may not adequately characterize global endothelial health for all populations. For instance, given the incidence of CVD following spinal cord injury (SCI), it may be surprising that normal brachial artery FMD has been reported (De Groot & Poelkens *et al.* 2004). The current authors previously reported decreased FMD in the legs (posterior tibialis) compared to the arms (radial) for patients with SCI (Stoner & Sabatier *et al.* 2006). The retention of upper-extremity function likely explains the lack of deterioration for SCI radial arteries. Patients with paraplegia mostly rely on upper-body function for performing daily activities. Since individuals with SCI actively use their upper extremities, blood flow patterns may be such that the blood vessels retain their functional status due to normal shear stressor activity (Zarins & Zatina *et al.* 1987; Gnasso & Carallo *et al.* 1996). Notably, we subsequently found that 18 weeks of self-administered neuromuscular electrical stimulation-induced resistance exercise therapy significantly increased FMD and arterial range of the posterior tibial artery in male patients with chronic, complete SCI (Stoner & Sabatier *et al.* 2007).

5. Limitations of the FMD test

Despite its potential, validity of the FMD test has been questioned due to lack of normalization to the primary stimulus (Mitchell & Parise *et al.* 2004; Pyke & Tschakovsky 2005; Stoner & McCully 2011). Despite the term *flow-mediated dilatation*, shear stress is the established stimulus for FMD (Koller & Sun *et al.* 1993). The magnitude of the shear stimulus created with reactive hyperemia is influenced by several factors; subsequently, the shear stimulus may differ significantly between individuals. Therefore, in order to efficaciously compare groups of individuals the shear stimulus should be considered (see below for further discussion). For instance, Mitchell *et al.* (Mitchell & Parise *et al.* 2004) demonstrated that reduced FMD may be attributable not only to impaired endothelial release of dilatory molecules, but also as a result of a lesser shear stimulus. Fortunately, the ultrasound technology used to conduct the FMD test can also provide estimates of shear stress.

6. Shear stress estimation

Clinical studies in humans, including FMD studies, typically estimate shear stress by employing a simplified mathematical model based on Poiseuille's law. More sophisticated approaches are available, but are beyond the reach of most clinical studies since such approaches are not readily available, too expensive, technically challenging and time-consuming (Oyre & Pedersen *et al.* 1997; Gatehouse & Keegan *et al.* 2005; Reneman & Arts *et*

al. 2006). Based on Poiseuille's law shear stress is calculated as the product of *shear rate* and blood viscosity, where shear rate equals:

$$\text{Shear rate } (\gamma) = \frac{2(2+n)v}{d} \quad (3)$$

where d is the internal arterial diameter, v is the time-averaged mean blood velocity, and n represents the shape of the velocity profile. For a fully developed parabolic profile, n is 2; this is the normal assumption when estimating shear rate.

Poiseuille's law assumes that: 1) the fluid (blood) is Newtonian, 2) blood flows through a rigid tube, 3) whole blood viscosity represents viscosity at the vessel wall and is linearly proportional to shear rate, 4) the velocity profile is parabolic, and 5) mean blood velocity adequately defines the shear stimulus. First, although blood is a non-Newtonian fluid at low shear rates (smaller than approx 100 reciprocal seconds (s^{-1})) (Chien & Usami *et al.* 1966) *in vivo*, shear rates in large arteries, particularly at the endothelial surface, are generally considerably larger than this threshold value so that the effect of the non-Newtonian behavior does not appear to be pronounced. Second, blood vessels are distensible, meaning that increases in arterial cross-section occur during the cardiac cycle. Wall shear rate may be ~ 30% less in a distensible artery as compared to a rigid tube (Duncan & Barger *et al.* 1990).

Third, to estimate shear stress from shear rate, invasive measures of blood viscosity are required. This potentially adds an additional source of error (Tangelder & Teirlinck *et al.* 1985). Human *in vivo* studies are usually limited to whole blood measurements of viscosity. These measurements overestimate the viscosity at the wall of the vessel. Less red blood cells travel along the artery wall, where, in addition to a thin layer of plasma, blood platelets are traveling (Tangelder & Teirlinck *et al.* 1985). Red blood cells tend to stream in the center of the vessel. The result is higher viscosity in the center of the vessel, thereby reducing the shear stress gradients at the vessel wall. It is worth noting that shear stress assessments do not seem to result in conclusions different from shear rate assessments alone (Padilla & Johnson *et al.* 2008). This may be explained by two factors: 1) sources of error from whole blood viscosity estimates, and 2) for a given population, viscosity changes little. Shear rate can therefore be used as an adequate surrogate measure (Gnasso & Carallo *et al.* 1996; Joannides & Bakkali *et al.* 1997; Betik & Luckham *et al.* 2004; Pyke & Dwyer *et al.* 2004; Padilla & Johnson *et al.* 2008).

Fourth, in arteries, the velocity profile will not form a well-defined parabola as a consequence of flow unsteadiness and short vessel entrance lengths. In both arteries and arterioles, the velocity profiles are actually flattened parabolas (Reneman & Arts *et al.* 2006). In the common carotid artery, mean wall shear stress is underestimated by a factor of two when assuming a parabolic velocity profile since the velocity difference is smaller between the innermost column of flow and the outermost circumferential layer (Dammers & Stiff *et al.* 2003). However, in the brachial artery - at least under baseline conditions - the underestimation is less pronounced - likely due to a more parabolic velocity profile in this artery (Dammers & Stiff *et al.* 2003).

Despite the aforementioned limitations, shear rate assessments can be reliably made using ultrasound (Samijo & Willigers *et al.* 1997). However, little attention has been given to the most appropriate blood velocity parameter(s) for calculating shear rate (see below).

6.1 Peak or integrated shear rates?

Recently, we conducted a study to determine which shear rate expression explained the most variation in FMD. Seven shear rate expressions were calculated: peak, change (peak hyperemia minus baseline), integrated over 10, 20, 30, and 40 seconds after tourniquet deflation, and integrated to peak diameter time (Stoner & McCully). Shear rates integrated for 40 seconds after tourniquet deflation (i.e., post-ischemia) explained the greatest portion of variation for change in diameter. However, the addition of *peak* shear rate to *time-integrated* shear rate was significant. This suggests that peak shear rate may be an additional important independent predictor of FMD. It is worth noting that while peak shear positively correlated with peak diameter when regressed independently, the addition of 40 seconds integrated shear rate led to a negative relationship between peak shear rate and peak diameter. A greater peak shear rate for a given integrated shear rate is indicative of a more transitory hyperemic response. A less sustained increase in shear rate may result in a lower stimulus mechanotransduced to the endothelial cells. This study was conducted on young, healthy males. Therefore, further study is warranted to confirm these findings on other cohorts.

6.2 Importance of the velocity profile

The earliest studies investigating the implications of shear stress on endothelial function did so by assessing endothelial cell responses to high versus low shear stress. This was until Davies *et al.* (Davies & Remuzzi *et al.* 1986), in 1986, provided evidence that the time-averaged shear stress alone could not explain the pathological behavior of endothelial cells exposed to complex flow patterns. Subsequent studies (Helmlinger & Geiger *et al.* 1991; Waters & Chang *et al.* 1997; Lum & Wiley *et al.* 2000; McAllister & Du *et al.* 2000; Peng & Recchia *et al.* 2000; Apodaca 2002; Blackman & Garcia-Cardena *et al.* 2002; Cullen & Sayeed *et al.* 2002; Barakat & Lieu 2003) have shown that vascular endothelial cells respond not only to the time-averaged shear stress, but respond differently to different patterns of flow.

The cyclic nature of the beating heart creates pulsatile flow conditions in all arteries. The heart ejects blood during systole, and fills during diastole. These cyclic conditions create relatively simple *mono-phasic* flow pulses in the upper region of the aorta (Wootton & Ku 1999). However, pressure and flow characteristics are substantially altered as blood circulates through the arterial tree. Figure 6 shows an example of a typical brachial artery blood velocity profile. The normal brachial arterial signal is *tri-phasic*, corresponding to 1) rapid blood flow during systole, 2) initial reversal of blood flow in diastole, and 3) gradual return of forward flow during late diastole.

The blood flow profile in the aorta is predominately governed by the force of blood ejected from the heart (Wang & Parker 2004). However, in the periphery the blood flow profile becomes more complex as a result of the energy transfer between the heart and arteries. The heart generates forward-traveling wave energy that propagates through the arteries to maintain tissue and organ perfusion for metabolic homeostasis. An individual forward-

traveling waveform, generated by the heart at the beginning of systole, initiates flow and increases pressure in the arteries. Although most of the wave energy in this initial compression wave travels distally into smaller arteries, some is reflected back towards the heart at sites of impedance mismatch. Interactions between forward- and backward-traveling waves result in complex blood flow patterns. Wave reflections result from arterial geometry, arterial wall compliance, and downstream resistance created by resistance arteries (Perktold & Rappitsch 1995; Barakat & Lieu 2003).

Complex flow characteristics have a profound impact on the shear stress distribution to which vascular endothelial cells are exposed. While human *in vivo* studies typically describe shear stress as a mean construct, numerous secondary phenomena associated with flow, including pulsatile flow, reversing flow, and flow turbulence, can influence the regulation of endothelial cells.

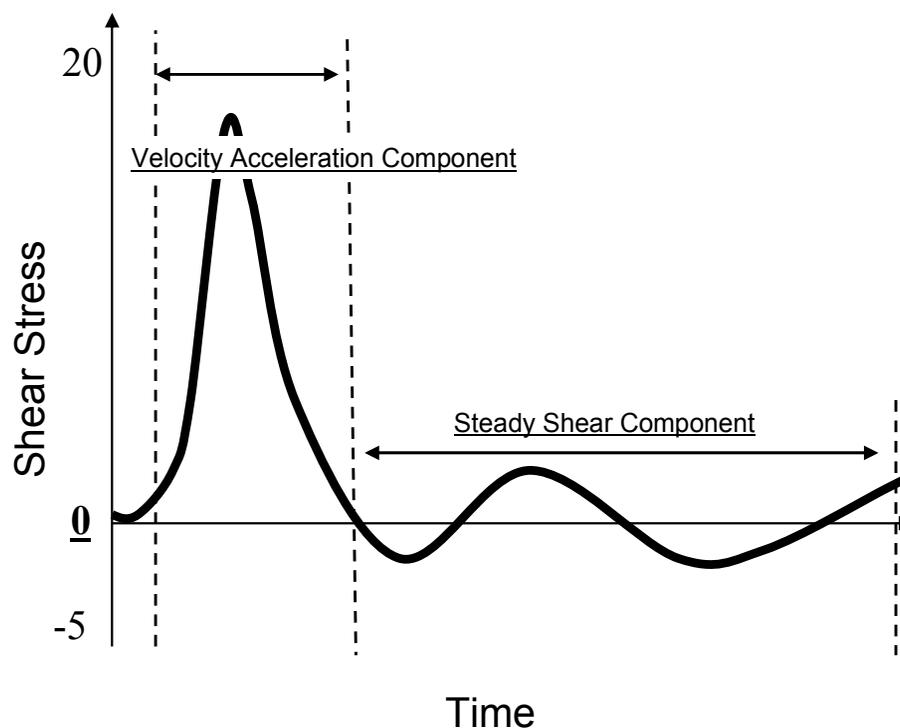


Fig. 6. Acceleration and steady shear components. The normal brachial arterial signal is tri-phasic, corresponding to the: 1) rapid blood flow during systole, resulting in velocity acceleration, 2) initial reversal of blood flow in diastole, and 3) gradual return of forward flow during late diastole, resulting in steady shear component.

6.2.1 Velocity acceleration and endothelial function

The pulsatile nature of blood flow exposes the endothelial cells to two distinct shear stimuli during the cardiac cycle: a large rate of change in shear at the onset of flow (velocity acceleration), followed by a steady shear component (Fig. 6). *In vitro* studies suggest that these two distinct fluid stimuli (velocity acceleration vs. steady shear) regulate short- and long-term endothelial function via independent biomechanical pathways (Ojha 1994; Bao & Lu et al. 1999; White & Haidekker et al. 2001; Hsiai & Cho et al. 2002 DePaola, 1992). Studies have shown that the rate of velocity acceleration can affect the progression of atherosclerosis

(Ojha 1994; Bao & Lu *et al.* 1999; Hsiai & Cho *et al.* 2001), endothelial cell function (White & Haidekker *et al.* 2001 ; Hsiai & Cho *et al.* 2002), mechanotransduction (Hsieh & Li *et al.* 1993; Bao & Clark *et al.* 2000), calcium kinetics (Helmlinger & Berk *et al.* 1995; Blackman & Barbee *et al.* 2000), and vascular tone (Frangos & Eskin *et al.* 1985; Noris & Morigi *et al.* 1995). Conditions which affect velocity acceleration include ventricular ischemia (Sabbah & Przybylski *et al.* 1987), acute myocardial infarction (Kezdi & Stanley *et al.* 1969), stenosis (Bassini & Gatti *et al.* 1982), hypertension (Sainz & Cabau *et al.* 1995), and hyperthyroidism (Chemla & Levenson *et al.* 1990). Velocity acceleration is also influenced by aging (Sainz & Cabau *et al.* 1995) and physical activity (Bonetti & Barsness *et al.* 2003; Shechter & Matetzky *et al.* 2003).

Recently, we studied the effect of velocity acceleration on FMD in a group of 14 healthy, young, male subjects (Stoner & McCully). FMD was measured prior to, and following, increases in velocity acceleration. Velocity acceleration (see Fig. 6) was increased by inflating a tourniquet around the forearm to 40 mmHg. We found that a 14% increase in velocity acceleration attenuated FMD by 11%. This finding suggests that mean blood velocity alone may not adequately characterize the shear stimulus. Attention to secondary flow phenomena may be particularly important when comparing groups with known secondary flow abnormalities.

6.3 Statistical analysis

Studies using the FMD test (i.e., using 5 minutes ischemia) should *consider* both time integrated- and peak-shear parameters, particularly when attempting to detect differences between experimental groups. Emphasis is placed on *consider* since the FMD test should not be normalized to shear rate using conventional approaches. A number of studies have attempted to account for the effect of shear stimulus on FMD by evaluating the quotient of FMD and shear, rather than FMD alone, or by using an analysis of covariance (ANCOVA), with shear stimulus as the cofactor (De Groot & Poelkens *et al.* 2004; Parker & Ridout *et al.* 2006; Padilla & Johnson *et al.* 2008; Atkinson & Batterham *et al.* 2009; Thijssen & Bullens *et al.* 2009; Pyke & Jazuli 2011). The techniques described above require use of the General Linear Model (GLM) for determining statistical probabilities associated with the differences found between groups or experimental treatments. However, when using a GLM the following assumptions must hold true: 1) there must be at least a moderate correlation between the two variables (i.e., shear and FMD), 2) the relationship between shear and diameter must be linear, 3) the intercept for the regression slope must be zero, 4) variance must be similar between groups, and 5) data must be normally distributed (Allison & Paultre *et al.* 1995; Atkinson & Batterham *et al.* 2009). A recent study found that all assumptions for reliable use of shear-diameter ratios were violated (Atkinson & Batterham *et al.* 2009).

Another alternative is to normalize the FMD response (i.e., change in diameter) to shear using hierarchical linear modeling (HLM) (Raudenbush & Bryk 2001). HLM is a more advanced form of multiple linear regression that accounts for hierarchical (i.e., successive inter-related levels) effects on the outcome variable. This is accomplished in HLM by including a complex random subject effect which can appropriately account for correlations among the data. This approach models different patterns in the data by allowing for the intercepts (initial diameter) and slopes (shear rate-diameter) to randomly vary. A third level

may also be specified; this may be the specification of groups (e.g., to delineate differences in endothelial function), an intervention or a modifiable risk factor such as smoking. This approach has been used to compare upper vs. lower extremity arterial health in persons with spinal cord injury (SCI) (Stoner & Sabatier *et al.* 2006), to assess improvements in arterial health following electrical stimulation-evoked resistance exercise therapy in persons with SCI (Stoner & Sabatier *et al.* 2007), and to assess the effects of occasional cigarette smoking on arterial health (Stoner & Sabatier *et al.* 2008). The disadvantage of this approach is that multiple stimuli (preferably ranging from minimal to maximal shear stimuli) are required to generate a reliable shear-diameter relationship.

7. Improving reliability of the FMD test

The within-subject variability of FMD has been reported to be as low as approximately 50% (De Roos & Bots *et al.* 2003), which helps to explain why FMD is related to low but not medium or high CVD risk (Witte & Westerink *et al.* 2005). Within any given study, FMD tests can consistently demonstrate a smaller degree of dilation in subjects with atherosclerotic/risk factors versus controls. However, subtle changes in FMD are more difficult to detect. Patients with only one CVD risk factor report FMD values of approximately 7% (Accini & Sotomayor *et al.*). For a typical brachial artery with a 4 mm diameter at baseline, this translates to a 0.28 mm increase in diameter. The pixel resolution of a typical ultrasound unit is 0.04 * 0.04 mm. Measurements of 0.28 mm are within the standard error of measurement. To compound the issue maximal diameters in response to reactive hyperemia are short lived and, therefore, hard to capture. Aside from standardizing measurement protocols, measurement reproducibility can be improved by considering the following suggestions.

7.1 Automate diameter measurements

Studies using edge detection software to automate diameter measurement when calculating FMD have reported intersession coefficients of variation of approximately 14-18% (Hijmering & Stroes *et al.* 2001; Woodman & Playford *et al.* 2001).

7.2 Use ANCOVA to account for measurable covariates

FMD can be calculated as: 1) post-only score, 2) change score, 3) fraction, or 4) co-varied for resting diameter. A simulation study found the greatest statistical power for the ANCOVA approach out of the four methods listed above, with fraction scores resulting in the lowest power (Vickers 2001). Expressing FMD as a percentage effectively squares the variation due to resting diameter, and may result in a non-normally distributed statistic from normally distributed data. Using resting diameters as a covariate is most likely to adjust for the bias due to baseline values (Vickers 2001; Twisk & Proper 2004; Tu & Blance *et al.* 2005)

7.3 Normalize to the stimulus

While FMD is certainly attenuated in a number of disease states, FMD may also be “attenuated” if the magnitude of hyperemia (Mitchell & Parise *et al.* 2004) or the blood velocity profile is altered, including the rate of velocity acceleration (Stoner & McCully).

Further study is required to comprehensively quantify the appropriate expression of the shear stimulus. At present, shear rate can be calculated as described above and used to normalize to FMD through HLM, but not by using ANCOVA or presenting as a ratio.

7.4 Use multiple FMD measurements when possible

The peak diameter in response to reactive hyperemia is short lived and, therefore, hard to capture (see Fig. 3). Variance in peak diameter measurements may be attributable to differences in the stimulus (i.e., shear stress) or to measurement error (see Fig. 7). Variance due to change in the stimulus can be accounted for by normalizing FMD to shear stress. To account for measurement error, according to laws governing regression to the mean (Shephard 2003), the FMD test would need to be repeated multiple times in order to obtain a “true” response. Alternatively, a more accurate assessment of endothelial function can be achieved by estimating shear rate-diameter dose-response curves (see Fig. 8).

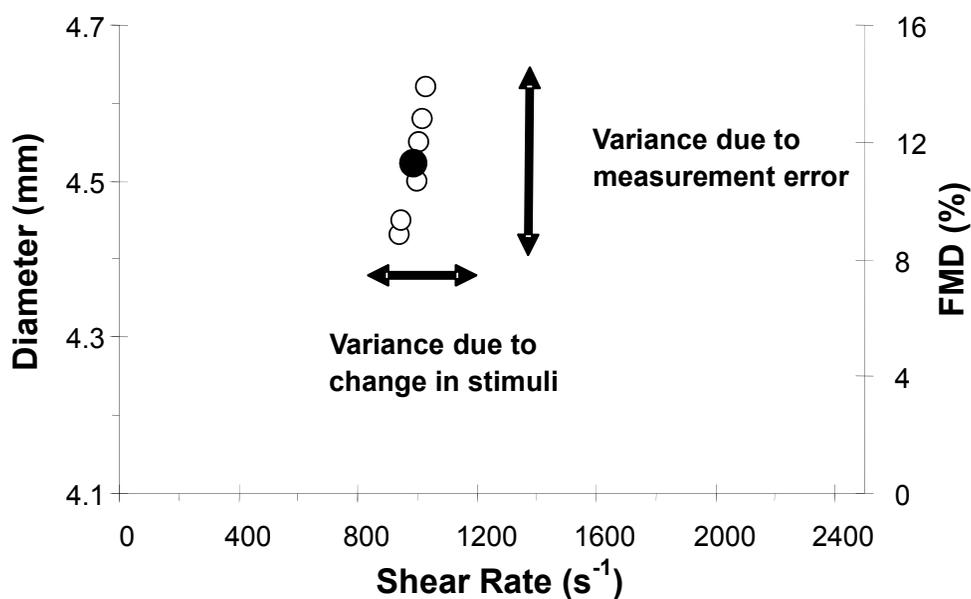


Fig. 7. Flow-mediated dilation (FMD) measurement variance. Open circles represent multiple FMD measurements. The closed circle represents mean FMD. Variance due to change in stimuli (shear rate) can be accounted for by normalizing to shear rate. Variance due to measurement error can be minimized by multiple FMD measurements (or by calculating a shear rate : diameter dose response curve).

7.5 Shear rate: Diameter dose response curves

Capturing shear rate-diameter dose-response curves (Stoner & Sabatier *et al.* 2004; Stoner & Sabatier *et al.* 2006; Stoner & Sabatier *et al.* 2007; Stoner & Sabatier *et al.* 2008) will decrease the likelihood of making erroneous conclusions. A standard dose-response curve (Fig. 8) is defined by three parameters: the baseline response (Bottom), the maximum response (Top), and the slope. The slope (i.e., change in diameter per one unit change in shear rate) would most accurately reflect endothelial function. The maximum response reflects the degree of arterial stiffness (Harris & Faggioli *et al.* 1995; Black & Vickerson *et al.* 2003; Sabatier & Stoner *et al.* 2006; Stoner & Sabatier *et al.* 2006).

There are a number of advantages to this approach, namely: 1) the stimulus (shear) is directly accounted for in a manner that does not violate statistical assumptions, 2) improved sensitivity, i.e., the slope (endothelial function) can be clearly identified (with the standard FMD test it cannot be ascertained at which point on the slope endothelial function is being estimated), 3) improved reliability, i.e., the dose-response slope is more resistant to measurement error when compared to a single measurement (Shephard 2003), and 4) more information is provided, i.e., the slope isolates endothelial function whereas the maximum response more likely reflects the degree of arterial stiffness (Harris & Faggioli *et al.* 1995; Black & Vickerson *et al.* 2003; Sabatier & Stoner *et al.* 2006; Stoner & Sabatier *et al.* 2006).

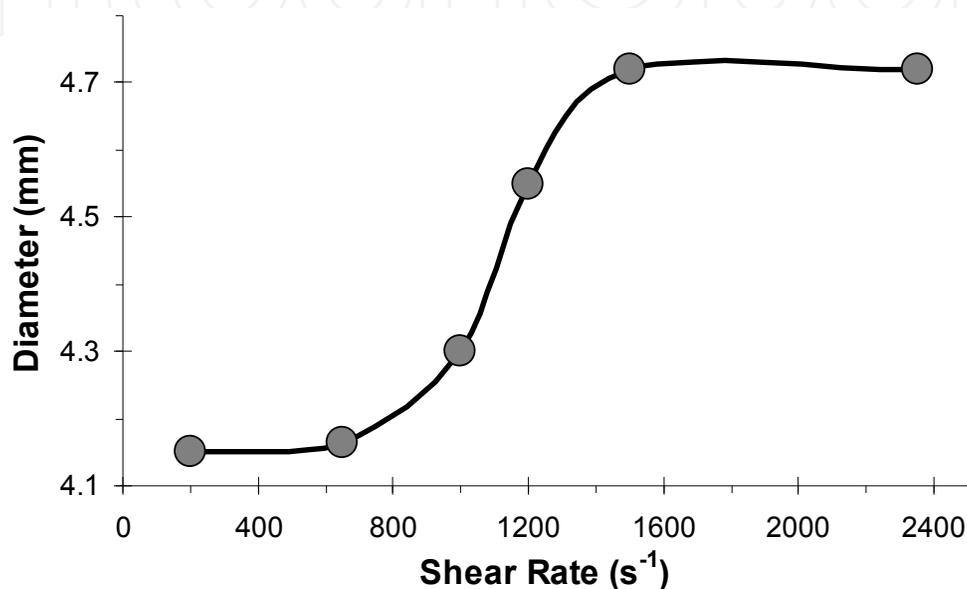


Fig. 8. Theoretical shear rate-diameter dose-response curve. Six data points are shown: baseline, and the responses to 5 durations of ischemia.

7.6 Transient versus steady-state shear stress

To overcome the short lived reactive hyperemia response, and hence short lived change in diameter, endothelial function can be evaluated by using sustained increases in shear stress, e.g., through local hand warming and low-intensity handgrip exercise (Mullen & Kharbanda *et al.* 2001; Joannides & Costentin *et al.* 2002; Pyke & Dwyer *et al.* 2004; Stoner & Sabatier *et al.* 2004). This approach would also allow for more accurate assessment of shear rate since the assumptions of Poiseuille's law are less likely to be violated (full description provided above).

Recently, we found that the relationship between shear rate and vasodilatation is comparable when shear rate is increased transiently (ischemia-induced) or in a sustained manner (local hand warming- and handgrip exercise-induced) (Stoner & McCully). This is consistent with a recent study by Pyke *et al.* (Pyke & Jazuli 2011), who similarly found a significant relationship between ischemia-induced FMD and handgrip exercise-induced FMD when the FMD responses were normalized to shear rate. Consideration has to be given to the mechanism(s) inducing FMD; the mechanisms regulating vascular tone may be dependent on the duration of the shear stimulus (Frangos & Eskin *et al.* 1985; Macarthur &

Hecker *et al.* 1993; Kuchan & Jo *et al.* 1994; Frangos & Huang *et al.* 1996; Mullen & Kharbanda *et al.* 2001), with FMD in response to sustained shear rate likely being less NO-dependent (Doshi & Naka *et al.* 2001). Nonetheless, the endothelium is still thought to primarily govern vasodilation under steady-state shear rate conditions. For instance, studies have shown that hand warming has no effect on brachial artery diameter when flow is not allowed to rise (Joannides & Bakkali *et al.* 1997; Mullen & Kharbanda *et al.* 2001; Pyke & Dwyer *et al.* 2004). Furthermore, pharmacological blockade of the autonomic nervous system has no effect on radial artery FMD in response to hand warming (Mullen & Kharbanda *et al.* 2001), consistent with animal studies showing that FMD is preserved after surgical or pharmacological denervation (Hilton 1959; Lie & Sejersted *et al.* 1970).

A recent meta-analysis by Inaba *et al.* (Inaba & Chen *et al.* 2010), which was subsequently re-analyzed by Green *et al.* (Green & Jones *et al.* 2011), assessed the CVD prognostic strength of FMD by conducting a meta-analysis of observational studies which examined the association between brachial artery FMD and future cardiovascular events. Green *et al.* found that FMD resulting from more intense and prolonged shear stimuli using proximal cuff placement, which has been demonstrated to be less NO-dependent (Doshi & Naka *et al.* 2001), provides a better prognosis for CVD risk. Further study is needed to confirm these findings and determine whether FMD in response to sustained increases in shear rate provides greater prognostic strength for detecting future CVD events.

8. Conclusions

Assessments of endothelial function offer the potential to predict and track individuals at risk for CVD complications. However, despite the obvious potential, the reliability of this test has been questioned. Recently, a range of practices has been adopted to improve test reliability, including consideration of the shear stimulus and automated diameter measurements. However, the standard approach for inducing the shear stimuli, i.e., reactive hyperemia following ischemia, has inherent limitations, namely: 1) the peak diameter in response to reactive hyperemia is short lived and, therefore, hard to capture, and, 2) there is no consensus on the appropriate calculation of shear stress/rate. These limitations can be overcome with the following strategies: 1) repeat the FMD test multiple times to obtain a more reliable estimate of the “true” response, 2) calculate the shear rate-diameter dose-response to decrease the likelihood of erroneous conclusions, and, 3) use sustained increases in shear stress. Further study is required to determine: 1) whether shear-rate diameter dose-response curves offer greater statistical power, 2) whether FMD in response to sustained increases in shear rate provides greater prognostic strength, and, 3) the importance of secondary flow phenomena to estimations of shear rate.

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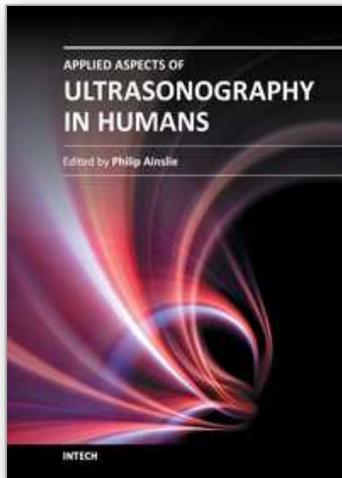
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