



# Overview of the Assessment of Endothelial Function in Humans

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The endothelium is recognized to play an important role in various physiological functions including vascular tone, permeability, anticoagulation, and angiogenesis. Endothelial dysfunction is increasingly recognized to contribute to pathophysiology of many disease states, and depending on the disease stimuli, mechanisms underlying the endothelial dysfunction may be markedly different. As such, numerous techniques to measure different aspects of endothelial dysfunction have been developed and refined as available technology improves. Current available reviews on quantifying endothelial dysfunction generally concentrate on a single aspect of endothelial function, although diseases may affect more than one aspect of endothelial function. Here, we aim to provide an overview on the techniques available for the assessment of the different aspects of endothelial function in humans, human tissues or cells, namely vascular tone modulation, permeability, anticoagulation and fibrinolysis, and the use of endothelial biomarkers as predictors of outcomes.

**Keywords:** endothelial dysfunction, diagnostic markers, biomarkers, microvascular, macrovascular, vascular tone, anticoagulation, permeability

## INTRODUCTION

The endothelium consists of a single cell layer of endothelial cells, lining the vascular and lymphatic systems, and covers a surface area of more than 1,000 m<sup>2</sup> (1). The apical surface of the endothelium is covered by a layer called the endothelial glycocalyx (2), which consists of a mosaic of glycoproteins and proteoglycans, and glycosaminoglycan chains. The endothelial glycocalyx, together with secreted proteoglycans and other adsorbed plasma proteins including albumin, forms the endothelial surface layer (3).

Endothelial cells from different vascular sites and organs have demonstrated variations in their appearance and surface constituents (4–6). Likewise, the corresponding endothelial glycocalyx have also been shown to be different in different organ beds (7). This phenotypic heterogeneity allows the endothelium to serve an array of functions tailored for different sites.

In the 1950s, the endothelium was believed to be merely a “sheet of nucleated cellophane” (8), however the importance of the endothelium in haemostasis and arterial smooth muscle relaxation, and thus vascular tone, was subsequently recognized (9, 10). The term “endothelial dysfunction” was then coined following the observation of diminished pulmonary exchange after intratracheal administration of bleomycin in rabbits (11). Currently, the endothelium is known to play an important role in the modulation of vascular tone, dynamic permeability, thrombogenicity, inflammation, and angiogenesis (12–14). These functions are achieved via receptors and release of a diverse family of compounds such as autacoids like prostacyclin, endothelin-1, and angiotensin II (13).

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Given the diversity in function and heterogeneity of the endothelium, measurement of endothelial function is challenging. Endothelial function in certain aspects are studied in greater detail compared to others due to earlier understanding of certain physiology and the availability of technology. For example, endothelial function in terms of angiogenesis is not well-studied *in vivo* in humans, although there are various assays and guidelines on *in vitro* interpretation (15, 16), while endothelial function in terms of vascular tone modulation has been much more widely studied (17).

In this review of endothelial function measurement, we will be concentrating on areas where techniques to measure endothelial function in humans or human cells and tissues are well-established. The sections are categorized into: (1) vascular tone modulation and tissue perfusion, (2) dynamic permeability, (3) anticoagulation and fibrinolysis, and (4) endothelial function biomarkers. Each section is then further categorized into broad physiological phenomena utilized (if relevant), then subcategorized into *in vivo*, *ex vivo*, or *in vitro* techniques and then the method itself. We will summarize various techniques used to quantify endothelium function, the underlying evidence, and clinical associations reported. Many of the existing techniques measure surrogate endpoints, as we are unable to directly measure endothelial function. Techniques detailed in the same section should thus, not be treated as equivalent to each other (18, 19).

We categorized the methods according to the endothelial function being assessed and underlying physiological principle utilized, for ease of reference, although certain methods may be able to assess more than one function of the endothelium. As this review is meant to be an overview of methods available to measure endothelial function, the physiological basis behind the measurements and limitations will only be briefly mentioned (Table 1).

## VASCULAR TONE MODULATION AND TISSUE PERFUSION

In healthy vessels, the endothelium regulates vascular tone locally via autacoids including prostacyclin and nitric oxide (NO),

**Abbreviations:** AFM, Atomic force microscopy; Ang, Angiopoietin; ANGPTL, Angiopoietin like proteins; AT, Antithrombin; CEC, Circulating endothelial cells; CFC, Capillary filtration coefficient; DAF-2, 4,5-diaminofluorescein; EDD, Endothelium dependent dilation; ezFMD, enclosed zone flow mediated dilation; ELISA, Enzyme linked immosorbent assay; FITC, Fluorescein isothiocyanate; Flk-1, Fetal liver kinase-1 (also known as VEGFR-2); Flt-1, Fms-like tyrosine kinase-1 (also known as VEGFR-1); FMD, Flow mediated dilation; FXa, Factor Xa; ICAM-1, Intercellular adhesion molecule-1; IDF, Incident dark field; LC/MS, Liquid chromatography/tandem mass spectrometry; LDF, Laser Doppler flowmetry; LDI, Laser Doppler imaging; LDPI, Laser Doppler perfusion imaging; L-FMC, Low-flow-mediated-constriction; MRI, Magnetic resonance imaging; NO, Nitric Oxide; OPS, Orthogonal polarization spectral; PECAM-1, Platelet endothelial cell adhesion molecule-1; PDGF, Platelet derived growth factor; PORH, Post-occlusive reactive hyperaemia; RHI, Reactive hyperaemia index; ROS, Reactive oxygen species; SDF, Sidestream dark field; TEER, Transendothelial electrical resistance; tPA, Tissue plasminogen activator; VCAM-1, Vascular cell adhesion molecule-1; VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor receptor; ZO-1, Zonula occludens-1.

which have been broadly categorized into endothelium-derived relaxing, hyperpolarizing and contracting factors (68). NO is a well-known and well-studied molecule that is predominantly characterized as an endothelium-derived relaxing factor (69). NO activates soluble guanylyl cyclase in vascular smooth muscle cells, resulting in cyclic guanosine monophosphate production and triggering relaxation (70). Stimulators for the release of endothelium-derived relaxing factors reported include serotonin, thrombin, adenosine, bradykinin, and insulin (71). The endothelium can also release endothelium-derived contracting factors such as vasoconstrictor prostanoids and endothelin-1, which stimulates the vascular smooth muscle cells to contract (71). Endothelium dependent dilation (EDD) is thus dependent on a multitude of factors, ranging from NO production, prostanoids, endothelin-1, and other endothelium derived hyperpolarizing factors (71).

Endothelial dysfunction in terms of vascular tone is categorized as any phenotype leaning toward a vasoconstrictive profile (72, 73) and may arise from reduction in production of relaxing mediators or altered endothelial response to relaxing mediators. Blunting of EDD is observed in patients who have traditional cardiovascular risk factors such as smoking (74) and is a predictor of future cardiovascular disease (75, 76). Overall vascular tone modulation or vasodilatory response to ischemic stimulation is a surrogate measure of endothelial function.

EDD is commonly used to determine endothelium dysfunction in vascular tone modulation. Besides measuring EDD response to direct chemical stimulus, physiological responses namely reactive hyperaemia can also be utilized. Post-occlusive reactive hyperaemia (PORH) describes the phenomenon where blood flow increases after a period of arterial avascularisation or ischaemia (77), and is in part, due to EDD depending on the vascular site analyzed. Characteristics of blood flow, including peak blood flow, is measured pre- and post-ischaemic challenge through inflation of a cuff at rest to temporarily stop blood flow or via exercise (17, 78, 79). Sites of measurements may include arteries, veins and capillaries using different methods as follows (17, 78, 79).

Other measures of vascular tone modulation besides measuring EDD include measuring arterial stiffness or compliance and analyzing the pulse wave characteristics (80). Arterial compliance can be quantified by the difference between change in vessel diameter in systole and diastole or pulse wave velocity. Potential sites of measurement include the brachial artery, carotid artery or aorta. Characteristics of the pulse wave including velocity can be quantified by pulse contour analysis. Pulse wave velocity is obtained by measuring the speed of the arterial pressure wave propagation and is inversely related to arterial wall stiffness.

## Endothelium Dependent Dilation and Others

### *In vivo* Techniques

#### *Intra-arterial or intra-venous infusion*

In the arteries, a few methods are established to measure EDD. One of them is intra-brachial infusion of acetylcholine,

**TABLE 1** | Summary of techniques in the measurement of endothelial function.

Technique	Invasiveness	Experiment type	Areas or parameters assessed	Specific limitations if any	Examples of clinical disease	Commercial devices available
<b>VASCULAR TONE</b>						
Intra-arterial or intra-venous infusion of vasoactive substances	Invasive	<i>In vivo</i>	Artery, venous, and microvasculature of study area	- Depending on vasoactive substance, and site of infusion, response measured may not be due to endothelium alone - Time consuming	Cardiovascular disease, smoking, hyperglycaemia, (20, 21)	Yes
<b>POST-OCCLUSIVE REACTIVE HYPERAEMIA</b>						
- Flow mediated dilation	Non-invasive	<i>In vivo</i>	Artery and microvasculature of study area (If using ultrasound: limited to forearm)	- If using ultrasound, high degree of operator skill required. - If using magnetic resonance imaging, it is expensive. - Strict protocol to follow.	Cardiovascular disease, hypercholesterolemia (22–24)	Yes
- Enclosed Zone Flow Mediated Dilation	Non-invasive	<i>In vivo</i>	Arteries of limbs	- Not as well-studied as flow mediated dilation	Cardiovascular disease (25)	Yes
- Laser based techniques	Non-invasive	<i>In vivo</i>	Skin microvasculature	- Laser required - Strict standardization of study site required.	Cardiovascular disease, diabetes (26–28)	Yes
- Digital reactive hyperemia index in peripheral arterial tonometry	Non-invasive	<i>In vivo</i>	Microvasculature of fingertips	- Disposable finger plethysmographic probes required, which can be costly	Cardiovascular diseases, hypertension, diabetes, smoking, infection, and autoimmune disease (29–33)	Yes
- Venous occlusion plethysmography	Non-invasive	<i>In vivo</i>	Venous system of limbs	- Strict protocol to follow.	Cardiovascular disease, vascular claudication, diabetes (34–36)	Yes
<b>ARTERIAL STIFFNESS</b>						
- Arterial compliance, pulse wave analysis	Non-invasive	<i>In-vivo</i>	Arteries	- Strict protocol to follow.	Cardiovascular disease, obesity, hypertension, smoking, diabetes (37–39)	Yes
- Atomic force microscopy	Invasive	<i>Ex-vivo</i>	Endothelial glycocalyx	N. A.	Cardiovascular disease, diabetes, infections, inflammatory disease, cancer (40–42)	Yes
- Myograph	Invasive	<i>Ex-vivo</i>	Arteries	- Opportunistic sampling	Cardiovascular disease, pre-eclampsia (43–45)	Yes
- Endothelial cell culture	N.A.	<i>In vitro</i>	- Cellular nitric oxide release - Flow-induced nitric oxide release	- Important <i>in vivo</i> characteristics may be lost, however this may be modulated with microfluidic models	Cardiovascular disease, diabetes, hypertension, pulmonary arterial hypertension (46, 47)	Yes
- Intra-vital microscopy e.g., OPS, SDF or IDF	Non-invasive	<i>In-vivo</i>	Microvasculature, endothelial glycocalyx	N. A.	Cardiovascular, infection, pulmonary arterial hypertension (48–50)	Yes
- Microdialysis	Invasive	<i>In-vivo</i>	Microvasculature	N. A.	Infection (51)	Yes
<b>DYNAMIC PERMEABILITY</b>						
- Venous occlusion plethysmography	Non-invasive	<i>In vivo</i>	Capillaries	- Strict protocol to follow.		Yes
- Transendothelial electrical resistance	N.A.	<i>Ex vivo—In vitro</i>	Ionic conductance of paracellular pathway	- Cultured endothelial monolayer in the absence of flow shear stress have impaired barrier function, however this may be modulated with microfluidic models	Neurological disorders Infection, inflammatory disease (52–55)	Yes

(Continued)

TABLE 1 | Continued

Technique	Invasiveness	Experiment type	Areas or parameters assessed	Specific limitations if any	Examples of clinical disease	Commercial devices available
- Transendothelial flux of labeled macromolecules	N.A.	<i>Ex vivo—In vitro</i>	Paracellular water flow and pore size of tight junction	- Cultured endothelial monolayer in the absence of flow shear stress have impaired barrier function, however this may be modulated with microfluidic models	Diabetes, pre-eclampsia (56–58)	Yes
<b>ANTI-COAGULATION AND FIBRINOLYSIS</b>						
- Tissue plasminogen activator release	Invasive	<i>In vivo</i>	tPA release from forearm vasculature	N.A.	Cardiovascular disease, hypercholesterolemia, hypertension, smoking (59, 60)	Yes—Assay to measure tissue plasminogen activator
- Functional assay to neutralize FXa and thrombin	Invasive	<i>Ex vivo—In vitro</i>	Ability to neutralize FXa and thrombin	N.A.	Autoimmune disease (61, 62)	Yes—Assay to measure FXa and thrombin
<b>OTHERS</b>						
Endothelial function biomarkers	Non-invasive to Invasive	<i>Ex vivo</i>	Systemic vasculature including endothelial glycocalyx	- Kinetics may not be well-known	Cardiovascular, infection, cancer, autoimmune, pre-eclampsia, and eclampsia (63–67)	Various ELISA kits may be available

which evokes EDD measurable by ultrasound (81). Acetylcholine is the most frequently prescribed endothelium-dependent vasodilators in experimental models and human research studies. Acetylcholine binds to muscarine receptors and activates endothelial NO synthase, stimulating the production of NO from L-arginine. Other NO agonist such as methacholine, and bradykinin can also be used (82). Intra-arterial infusion of other vasoactive substance can also be used to measure endothelial reaction, and thus dysfunction if reaction is impaired.

Besides intra-arterial infusion, intravenous infusion of vasoactive compounds can also be performed to assess endothelial function (83). Dorsal hand vein technique can be used to determine venous tone and was first described in 1981 (84). A needle is inserted into a vein on the hand dorsum and a linear transformer is connected to the needle to measure vein diameter after pre-treatment with vasoactive compounds. Depending on the vasoactive substance infused, underlying physiological reactions may be different and may not be purely due to EDD. While intra-arterial and intra-venous infusion methods are fairly safe, they are still invasive, and more time-consuming than other methods available (82).

### Flow mediated dilation

Flow mediated dilation (FMD) is a common method used to measure vascular endothelial function of the brachial artery and utilizes imaging, commonly ultrasound, to measure the arterial dilation during PORH. PORH can be achieved thorough inflating a pneumatic pressure cuff around the arm to suprasystolic pressure for 5 min. Reactive hyperaemia is then generated

by rapidly releasing the pressure and in the brachial artery, this phenomenon is due to NO-mediated EDD. The brachial artery is thus a favored site for measuring FMD as NO is the sole mediator of FMD in the brachial artery, and provides a more accurate surrogate measure of endothelial NO production (85). Impaired dilation suggests impaired release of endogenous vasodilator in response to ischemia and thus endothelial dysfunction.

Maximal artery diameter is recorded and the FMD%, an index, is then calculated from the pre- and post-occlusion maximal artery diameter (22, 86). Hyperaemic blood flow velocity can also be calculated in the same setting (87, 88), however scores obtained from measuring blood flow velocity e.g., pulsatility index or resistance index are not as well-studied as compared to FMD for measurement of endothelial function (89, 90). Arterial stiffness can also be calculated and is elaborated in the corresponding section below.

The use of ultrasound for FMD limits its use to easily visualized arteries, requires a high frequency transducer, and skilled ultrasonologist for accurate measurements. Otherwise, large errors in flow estimation can occur (91). A standardized protocol on subject preparation and assessment of FMD is also required to ensure reproducibility (86, 92, 93). Other methods to measure FMD includes magnetic resonance imaging (MRI), and this has been validated against ultrasound (94). Use of MRI allows PORH of deeper arteries to be visualized and limits inter-observer differences but requires the use of more expensive equipment and highly skilled personnel, which may not be practical in low resource settings.

New techniques including enclosed zone FMD (ezFMD) have been developed to circumvent some of the abovementioned limitations. In ezFMD, oscillometry is used to measure changes in oscillation amplitude of intra-arterial pressure changes and thus variation in vascular volume (25). Change in vascular dilatation in response to ischaemia can potentially quantify lower-limb vascular endothelial function. As oscillometry is used, ezFMD readings can be obtained using an air cuff with minimal technical expertise and invasiveness.

On a side note, low-flow-mediated-constriction (L-FMC) can be measured in the same setting as FMD and provides additional information beyond FMD on endothelial dysfunction (95, 96). L-FMC involves measuring the diameter and flow in the last segment of arterial avascularization and thus vascular reactivity. The measured L-FMC is likely to be due to a combination effect of both endothelial derived relaxing and contracting factors and not EDD alone. L-FMC is expressed as the percentage of the decrease in diameter during arterial avascularization with resting diameter (95, 96).

#### **Reactive hyperaemia index—peripheral arterial tonometry**

PORH has also been studied in microvasculature and is measured as reactive hyperaemia index (RHI) and was first developed in 2003 (97). EDD is measured by the digital pulse waveform, also known as peripheral arterial tone. Disposable modified plethysmographic probes on the index fingers measure the digital pulse waveform pre and post-occlusion of the brachial artery (98). Pre-and-post-occlusion values are then used to calculate the RHI. A linear relationship between RHI and FMD was demonstrated and further studies and a meta-analysis has demonstrated association with cardiovascular events (99).

#### **Venous occlusion plethysmography**

The use of venous occlusion plethysmography to measure blood flow was first described in the 1900s (100). Venous return from the limb or area of study is interrupted via inflation of a cuff to below diastolic pressure, allowing for arterial inflow and venous emptying. Blood flow is then measured by change of volume over a fixed duration. The maximal blood flow is influenced by vascular resistance, and thus vascular tone. Venous occlusion plethysmography therefore can be used to indirectly measure local vascular tone in response to an ischaemic challenge. Change in limb volume can be measured via air filled, water filled, mercury plethysmography or impedance plethysmography (101–104). Additional modifications to this technique include combination with intra-arterial drug administration and the use of automation. While the results appeared repeatable and reproducible, variation in factors such as cuff inflation time may significantly affect blood flow recorded (105).

#### **Laser Doppler**

Laser Doppler flowmetry (LDF) is another non-invasive technique used to measure EDD of skin microvasculature post stimuli (106). The laser light penetrates skin and soft tissue and is then partially backscattered by red blood cells. Based on the Fizeau Doppler principle, blood flow velocity is then derived from the frequency of the backscattered light (107).

The first developed laser Doppler technique was flowmetry, otherwise known as Doppler perfusion monitoring, where skin microvasculature was assessed over a small volume of maximum 1 mm<sup>3</sup>. However, due to regional heterogeneity of skin microvasculature and the small volume assessed, the reproducibility is poor (108). With improvement in technologies, laser Doppler imaging (LDI) or laser Doppler perfusion imaging (LDPI) has allowed for a larger volume of interest to be imaged and quantified (106, 109), this may result in improvement in reproducibility. Laser speckle contrast imaging (LSCI) utilizes a widespread laser, forgoing a grid scanning process required in the previous techniques, allowing faster imaging (110, 111). A range of stimuli can be used to stimulate the endothelium of the skin microvasculature as well, including PORH, local thermal hyperaemia, iontophoresis or transdermal application of acetylcholine, and microdialysis to introduce drugs of interest (109, 112, 113). Laser-based techniques have also been used in clinical use where assessment of microcirculation is warranted, such as in wound and burn assessments (114).

### **Ex vivo and in vitro Techniques**

#### **Endothelial cell culture**

Culture of human endothelial cells was described in 1960s (115) and has been used for high spatiotemporal imaging of intracellular processes and analyses of signaling molecules without background noise from other cell types (116). Direct measurement of mediators of specific stimuli on mediators of vascular tone, specifically NO release or reactive oxygen species can be performed via endothelial cell culture using different assays (117). An example would be the commonly used 4,5-diaminofluorescein (DAF-2) and cell permeable DAF-2 diacetate assay (117, 118), which quantifies real-time NO release from human endothelial cells.

### **Arterial Stiffness**

#### **In vivo Techniques**

#### **Arterial compliance and pulse wave analysis**

The use of blood vessel imaging with ultrasound or magnetic resonance in measuring FMD allows the measurement of arterial compliance with sonographic stiffness indices such as augmentation index (94, 119). Pulse wave velocity also allows measurement of arterial stiffness, with carotid-femoral pulse wave velocity being the gold standard for large artery stiffness (120). One-point pulse wave velocity and oscillometric methods have also been developed to measure arterial compliance easily (121, 122). Arterial stiffness is generally increased in persons with cardiovascular risk factors and heart failure, however it is not uniform across all arteries (37). As routine clinical measurement is not practical, pulse wave velocity measurement is not yet recommended for routine practice (123). Improvements to pulse wave velocity is possible with the use of angiotensin converting enzyme inhibitors and angiotensin receptor blockers (124), however clinical implications of this improvement has not been well-demonstrated (125).



## Ex vivo Techniques

### Myography

*Ex vivo* methods such as myography has been developed to measure arterial stiffness in association to endothelial dysfunction. Wire myography in 1970s was developed to study contractile responses of small resistance arteries (126) and since then other modifications such as isobaric and isotonic conditions have been utilized to optimize experimental studies (127, 128). Human mesenteric microarteries mounted in myographs have demonstrated an association between age and presence of cardiovascular risk factors with EDD (129). However, in view of the invasiveness, sampling is often opportunistic in human studies or from subcutaneous fat (130, 131).

### Atomic force microscopy

Atomic force microscopy (AFM) nano-indentation technique developed in 1986 (132) has also been utilized to measure the thickness and stiffness of the endothelial glycocalyx (133) which can be a prognostic marker for endothelial dysfunction. A triangular cantilever with a spherical tip periodically indents the endothelium and a laser beam is then used to measure the cantilever deflection. The thickness of the endothelial glycocalyx is then calculated from the force on the cantilever, piezo displacement and deflection sensitivity. AFM can be used in *in vitro* or *ex vivo* experiments of blood vessel preparations (133, 134).

## Intra-Vital Microscopy

Direct visualization of the microvasculature using hand-held intravital microscopes at the bedside has also been used in recent years (135). Microvasculature function includes endothelial function and leukocyte-endothelium interaction (136). Stagnant flow, red blood cell flow velocity can be directly observed and glycocalyx thickness indirectly observed using this method. Orthogonal polarization spectral (OPS) imaging is considered the first generation of hand-held intravital microscopes and was developed in 1990s (137). However, due to motion-induced image blurring in OPS imaging, sidestream dark field (SDF) imaging was developed with improvements in capillary contrast and quality (138). Incident dark field (IDF) imaging was then introduced subsequently in 2015 with improved resolution and visualization of more capillaries (139). An excellent review on the technical aspects has been written by Massey and Shapiro (140). Intravital microscopy is predominantly performed in the sublingual area and involves gently holding a hand-held pen-like-camera onto the study area. Intra-vital microscopy has correlated the thickness of the endothelial glycocalyx with microvascular perfusion (141), and use in sepsis have demonstrated thinning of the endothelial glycocalyx to be independently associated with worse clinical outcomes (142–144).

## Microdialysis

Microdialysis can also be used for real time measurement of reactive oxygen species (ROS) in the extracellular environment as a function of endothelium. The technique involves inserting porous probes into target tissue and perfusing it with physiological saline, allowing real-time and continuous passive

diffusion of molecules from extracellular fluid into a dialysis bag. The use of a dialysis bag to collect small amounts of samples from interstitial tissue was first described in 1966 in animal brains (145), and has since been used in a variety of tissues to explore different molecular pathways (146). The collected microdialysate can then be assayed with enzyme linked immunosorbent assay (ELISA) or liquid chromatography/tandem mass spectrometry for various molecules (LC/MS) (147). In the setting of quantifying endothelial dysfunction, ROS levels in interstitial tissues can be an indirect measure of tissue perfusion, and studies have associated microvascular endothelial dysfunction with elevated ROS levels (148).

## DYNAMIC PERMEABILITY

Endothelial cells are connected to each other via intercellular junctions including tight junctions and adherens junctions (149). These intercellular junctions regulate passive diffusion and allow the endothelial layer to form a selectively permeable layer to maintain homeostasis. Transport of molecules smaller than 3 nm occurs paracellularly while transport of macromolecules such as albumin occurs transcellularly in physiological conditions (150). The endothelium of different organ beds has different barrier functions and techniques for measuring endothelial dysfunction are not universally applicable.

### In vivo Techniques

The use of venous occlusion plethysmography to quantify vascular permeability was picked up in the 1960s (151), as there were initial technological difficulties in measuring small blood-tissue fluid shifts due to changes in capillary permeability when the predominant volume changes during venous occlusion occurred intravascularly (152). Nevertheless, venous occlusion plethysmography has subsequently been used to measure the permeability of capillaries in the form of the capillary filtration coefficient (CFC) (151, 153, 154). CFC consists of two components; one is the hydraulic conductance of the blood vessel and second is the surface area. Compared to measurement of PORH, the use of venous occlusion plethysmography to measure CFC involves occlusion via multiple steps of small increases in cuff pressure rather than one single step. The small increases in cuff pressure diminishes the effect of PORH and allows the increase in limb volume with each increase in cuff pressure to be due to filling of capacitance blood vessels and fluid efflux from the microvasculature. CFC is then obtained via least squares fitting of the volume response after filling of capacitance blood vessels is completed (151). Although CFC has been described in various disease processes, care must be exercised in documentation site of measurement and cuff pressure increase increment to ensure reproducibility (155, 156).

### In vitro Techniques

#### Transendothelial Electrical Resistance

*In vitro* experiment using cultured endothelial cell monolayers allows for transendothelial electrical resistance (TEER) to be measured as a surrogate measure of the barrier function (52). TEER measures ionic resistance which consists of resistance

associated with paracellular ion transport and ion transport across apical and luminal cell membranes. TEER can be measured via Ohm's law method which involves endothelial cell monolayers being cultured on a semipermeable filter in a two-compartment chamber, with an upper compartment and a bottom compartment. Electrodes are then inserted into the upper and lower compartments and are separated by the cultured endothelial cell monolayer. A direct or alternate current is then applied across the electrodes and the resulting current measured. TEER is then calculated based on Ohm's law.

Impedance spectroscopy is another method used to measure TEER. A small alternate current is applied across the electrodes and the amplitude and phase response of the resulting current is then measured. TEER is then calculated using the characteristics of the resulting current and phase shift. An excellent review on TEER has been published by Srinivasan et al. (52). TEER pre- and post-stimulus is recorded and results are reported as a percentage of baseline (52). Although TEER has been used *in vivo* animal studies (157), this technique is currently too invasive in humans, and is thus characterized under *in vitro* techniques.

### Transendothelial Permeability

Transendothelial permeability of labeled large molecules, ranging from carbon-14 labeled sucrose to fluorescein isothiocyanate (FITC) labeled albumin or dextran, across endothelial monolayers is another commonly used method to measure permeability (158). Macromolecule diffusion across endothelial monolayers can also be quantified using enzymatic markers such as horseradish peroxidase (159, 160) or visualized using confocal microscopy or electron microscopy (161, 162).

Barrier function in cultured endothelial monolayers alone however are not fully representative of *in vivo* conditions (163). There is variation in the reported thickness of the endothelial glycocalyx in cultured endothelial cells and *ex vivo* tissue (164), although the differences may be contributed by different in techniques (165, 166). Flow shear stress has also been demonstrated to enhance barrier function of the endothelium (167, 168) and formation and maintenance of healthy endothelial glycocalyx is dependent on the presence of flow (169). Technological advancements have since allowed optimization of the microenvironment of endothelial cell cultures to better replicate *in vivo* conditions including the establishment of flow shear stress. These methods have been further improved to resemble physiological conditions using tissue-on-a-chip model and can be generally classified as two-dimensional microfluidic models, hybrid microfluidic models, three-dimensional templated models, or self-organization models (170, 171). Despite these advances, models matching physiological microenvironment are still unavailable (172).

### Ex vivo Techniques

The measurement of protein or albumin loss in the urine is actively used in clinical evaluation of endothelial dysfunction in terms of permeability. In other end organs, such as the lung, the amount of total protein concentrations or other labeled proteins in the bronchioalveolar lavage fluid can also be measured as surrogate markers to endothelial permeability (173). The analysis

of bronchioalveolar lavage fluid however requires bronchoscopy to be performed and is considered invasive.

Human vascular endothelial cells can also be extracted via J-wires, pulmonary artery catheter balloons and other organ endothelial cells via surgery such as during tumor resection and cultured to evaluate site-specific barrier function with different stimuli (174–177). For example, barrier dysfunction was demonstrated via leakage of Evans blue dye-albumin across a monolayer of cultured human lung microvascular endothelial cells but not human umbilical vein endothelial cells under experimental sepsis conditions (176). Protein expression on the surface of these endothelial cells and chemokines production can also be analyzed to evaluate endothelial dysfunction (178). The breakdown of endothelial glycocalyx also hampers the dynamic permeability of the endothelium and will be expounded upon in the body fluid biomarkers section below.

## ANTICOAGULATION AND FIBRINOLYSIS

Tissue plasminogen activator (tPA), a key enzyme in the fibrinolytic pathway, is released by the endothelium and protects against development of atheroma, activation of platelets and formation of thrombus (179). Techniques for quantifying the *in vivo* release of tPA from the endothelium in response to stimuli have been developed (180) and can function as a measure of anti-thrombotic function. The measurement of tPA release is usually performed in the same setting with venous occlusion plethysmography for forearm blood flow (181, 182). Drugs such as substance P are infused via brachial artery cannulation and the release of tPA in response is then measured in the venous blood sampled from the subcutaneous veins in the antecubital fossa (180). In persons with traditional cardiovascular risk factors such as older age coupled with sedentary lifestyle, smoking and coronary artery disease, the release of tPA is reduced (20, 59, 183).

Functional analysis of anticoagulant activity in terms of ability to inactivate Factor Xa (FXa) and thrombin *in vitro* on endothelial tissues or cultured cells can also be performed (61). The tissue or cells are incubated with a fixed volume of antithrombin (AT) for 5 min, allowing the activation of AT through binding of surface-exposed heparan sulfate. A fixed amount of FXa or thrombin is then added and incubated for 2 min to allow activated AT to bind and inactivate FXa or thrombin. The supernatant is then analyzed for free FXa or thrombin. The amount of free factor Xa or thrombin thus indirectly measures the anticoagulation function of the endothelium.

## ENDOTHELIAL FUNCTION BIOMARKERS

Several biomarkers associated with endothelial function with clinical correlates have been identified, for example, studies have correlated levels of angiotensin, selectins, and growth factors with severity of sepsis (63, 184, 185). Majority of the biomarkers can be measured with ELISAs or LC/MS. However, the kinetic data of these biomarkers for endothelial injury are not well-known, and as such studies of these

biomarkers may have superficially contradictory results and reproducibility may depend on severity and duration of illness during measurement (63).

Angiopoietins (Ang) 1 and 2 belong to the family of growth factors and are involved in angiogenesis, as well as other functions including vascular permeability (184). Levels of Ang 1 and 2 in plasma both correlate with mortality and disease severity in sepsis (64, 186, 187). Angiopoietin like proteins (ANGPTL) are a family of proteins, of which eight have been described (188). Like angiopoietins, ANGPTL regulates angiogenesis and some may exhibit other functions and may be potential biomarkers for endothelial function. For example, ANGPTL 2 is a circulating glycoprotein and a proinflammatory mediator (188). In a study involving Japanese adults, ANGPTL 2 was found to be independently associated with risk of diabetes (189), and is possibly a key molecule linking obesity with insulin resistance and subsequent occurrence of diabetes. ANGPTL 6 might also play a role in endothelial function as higher levels were associated with pregnancy-induced hypertension (190).

Selectins expressed on activated endothelial cells may also be shed and measured in circulation, reflecting endothelial injury, and thus endothelial function (191). Selectins are key adhesion molecules and modulate leukocyte movement (192). Similar to selectins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and platelet endothelial cell adhesion molecule-1 (PECAM-1) also modulate leukocyte adhesion and movement (63, 193). Studies have correlated increased levels of selectins, ICAM-1 and/or VCAM-1 with sepsis although there are contradicting studies (63).

Growth factors such as vascular endothelial growth factor (VEGF) and soluble VEGF receptor-1 (VEGFR-1), also known as Flt-1, can also be quantified in circulation. VEGF and its receptors stimulates endothelial cell migration, hyperpermeability, and angiogenesis (194) and levels of VEGF and Flt-1 are both elevated in diseases with endothelial dysfunction such as sepsis and pre-eclampsia (195–197). VEGFR-2, also known as fetal liver kinase-1 (Flk-1) or kinase domain-containing receptor can also be measured in the serum, and may be a promising biomarker for certain cancers (198, 199). Current available angiogenesis assays are mainly used to assess anti-angiogenic or pro-angiogenic factors and not endothelial function (16). Thus, measurement of circulating biomarkers may be the closest method to measuring endothelial function in terms of angiogenesis.

Platelet derived growth factor (PDGF) also has multiple functions (200) and low levels can result in increased permeability (201). There are other potential biomarkers such as plasma soluble endoglin, a receptor of transforming growth factor-beta, which requires further evaluation. Levels of plasma soluble endoglin directly correlates with complicated diabetes and hypertension with end-organ damage, higher 10 year cardiovascular risk scores (202), pre-eclampsia (65), and yet an inverse correlation with severity of coronary artery disease (203).

Quantification of breakdown products of the endothelial glycocalyx layer has also gained interest in recent years.

Breakdown products of the endothelial glycocalyx layer has been shown to correlate with endothelial dysfunction in metabolic diseases (204, 205) including inborn errors of metabolism (206), infections such as malaria, dengue and sepsis, and acute respiratory distress syndrome (207–210). Measurable endothelial glycocalyx breakdown products in the plasma or serum include syndecan-1, chondroitin sulfate, dermatan sulfate, serum hyaluronic acid, and heparan sulfate (208, 211).

Syndecan-1 was the first proteoglycan characterized (212), and is a cell surface heparan sulfate proteoglycan that is released in endothelial glycocalyx damage (213). Syndecan-1 levels measured in circulation were associated with severity of sepsis, acute kidney injury, need for intubation, and mortality (214–216). Endocan, a soluble dermatan sulfate proteoglycan in endothelial cells, can also be measured in circulation (217). Levels of endocan correlated with severity of sepsis and mortality (218) as well as cancer (219, 220).

Total sulphated glycosaminoglycans can also be measured in the urine based on the reaction of 1,9-dimethylmethylene blue with sulphated glycosaminoglycans. Measurement of specific constituents e.g., heparan sulfate can also be measured by degrading heparan sulfate with nitrous acid prior. Other glycosaminoglycans e.g., chondroitin and dermatan sulfate will not be degraded by nitrous acid. Urinary heparan sulfate would then be the difference between sulphated glycosaminoglycans with and without nitrous acid treatment. LC/MS allows for quantification of specific glycosaminoglycans as well, including dermatan sulfate, heparan sulfate, and keratan sulfate (206).

Components of tight junctions can also be measured in the plasma and damage to endothelial barrier function quantified. A well-written review by Vermette et al. (221) summarizes the studies on endothelial injury and tight junction proteins. For example, claudin-5 is expressed mainly in vascular endothelial cells and modulates the permeability of tight junctions (222). Levels of claudin-5 in serum have been demonstrated to correlate with severe plasma leakage in dengue infection (223). On the other hand, levels of vascular endothelial cadherin, also known as cadherin-5, a main component of adherens junction, did not demonstrate any association with endothelial permeability (223) although *in vitro* study demonstrated loss of vascular endothelial cadherin resulted in increased permeability (224). Other tight junction proteins such as zonula occludens 1 (ZO-1), can also be measured in human plasma, and was found to be associated with inflammation and cancer (225, 226). ZO-1 however, is also expressed in other cells and may not reflect endothelial injury alone.

Circulating endothelial cells (CECs) were first described in peripheral blood films (227) and higher levels of CECs were then observed in people with cardiovascular risk factors and acute myocardial infarction (228). CECs originate from the mature endothelium and can be considered as markers of endothelial injury and thus function (229). The phenotype and morphology of CECs varies widely depending on the underlying disease (230–232). Identification and differentiation of CECs from other cells namely endothelial progenitor cells (EPCs) is also challenging. EPCs are believed to originate from the bone marrow and are incorporated into new blood vessels at sites of ischemia



and may function as part of an endogenous repair mechanism, rather than endothelial function itself (233–235). Improved characterization of CECs have allowed for immunomagnetic isolation and fluorescence activated cell sorting (FACS) to be used in identification of CECs (236) but further studies are needed to confirm that these methods are reproducible and accurate (237).

Endothelial microvesicles are small membranous particles released from endothelial cells and can be identified and measured from plasma through an array of methods including flow cytometry, ELISA, fluorescence confocal microscopy and electron microscopy (238). Clinical correlates with different types of endothelial dysfunction (238) and different triggers including infections, cancers and autoimmune conditions with endothelial microvesicles have been described (239). Further studies are also needed to better delineate the role of endothelial microvesicles in pathogenesis and thus its role in diagnostics and prognostication and hopefully therapeutics.

## CONCLUSION

Despite the wide array of techniques possible, our understanding of the human endothelium system is still not fully complete. The phenotypic heterogeneity of the endothelium cautions against extrapolating the physiology of endothelium in one organ bed to another organ bed. Invasive methods may still have to be utilized in organ beds that are not easily accessed or visualized and is likely not feasible in humans. Likewise, biomarkers measured in plasma may not reflect the local tissue environment and while microdialysis can measure the local tissue environment, it is still considered invasive and limited to accessible organs such as the skin. Specific limitations for each broad technique is described further in **Table 1**.

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For the assessment of pre-clinical disease, the ideal technique should be non-invasive, accurate, reproducible, low-cost, and easily perform (240). Several techniques described above still require further evaluation to optimize and standardized protocols to ensure reproducibility, while some may benefit from a meta-analysis to ensure that measurements correlate with clinical end points. We are also in need of a prospective trial comparing the various techniques quantifying endothelial dysfunction and eventual clinical endpoints. It is unlikely that one technique is superior than the rest, but perhaps a combination of techniques will allow for a comprehensive evaluation of endothelial function with our current technological capabilities.

## AUTHOR CONTRIBUTIONS

PC and TY conceptualized the review. PC drafted the manuscript and table which is original. PC, AT, and TY revised the manuscript critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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