Review

# Testing Endothelial Function and its Clinical Relevance

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**Endothelial dysfunction as an integrating index of the risk factor burden and genetic susceptibility is an early marker of atherothrombotic disease. Therefore, tremendous interest exists in its measurement and determination of the clinical utility of the evaluation of endothelial function.**

**Different invasive and non-invasive techniques exist for exploring various aspects of the pathobiology of the endothelium. As endothelial dysfunction is a diffuse-systemic disorder, the peripheral arteries, because of their accessibility, represent the basis for assessment of endothelial dysfunction. Flow-mediated dilation (FMD) of the peripheral conduit arteries is one of the most widely used tests of endothelial function. FMD measures the endothelial vasomotor response during reactive hyperemia, but it does not provide information concerning the control of arterial tone at rest. A new technique, low-flow-mediated constriction (L-FMC), provides complementary information to that by FMD, quantifying the decrease in the forearm conduit artery diameter that occurs in response to the decrease in blood flow during occlusion. This indicated that the L-FMC response is not based on nitric oxide availability but it might be mediated by other substances, providing a coordinated effect of vasodilation and its inhibition; therefore, simultaneous determination of FMD and L-FMC may provide comprehensive information on vascular homeostasis.**

**Peripheral arterial tonometry (PAT) evaluates pulse wave amplitude, which is linked to endothelial function. Like FMD, PAT has also been shown to be reduced in the presence of risk factors, as well as in patients with atherosclerosis; however, FMD of the brachial artery and PAT are very different methods for identification of the vascular reactivity of different arterial territories. FMD directly registers the dilation capability of the large-conduit artery, whereas PAT measures flow response hyperemia, which is related to the endothelial function of small arteries and to the endothelial function of the microcirculation. Therefore, this technique is mostly used for investigation of the functional capability of the microcirculation.**

**Determination of venous endothelial dysfunction is more complicated and invasive and is less reproducible. Micro-invasive techniques such as the dorsal hand vein technique and radionuclide assessment of changes in volume of the legs provide limited information about venous endothelial health; however, as endothelial dysfunction is expected to be a systemic disorder affecting the complete circulatory system, determination of the endothelial function of peripheral arteries also gives insight into venous functional status.**

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## Introduction

The endothelium has been recognized as the key regulator of vascular homeostasis. Healthy endothelium produces a wide range of factors that regulate vascular tone, adhesion of circulating blood cells to the vessel wall, thrombus formation, smooth muscle cell proliferation, and vessel wall inflammation, which is the key mechanism of the atherosclerotic process. One of the most important functions of the endothelium is its effect on vascular tone. This is achieved by the production of several vasoactive substances that

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relax or constrict the vessel, as well as by its response to circulating vasoactive mediators such as bradykinin and thrombin[1]. Nitric oxide (NO) has the most expressed vasodilating capacity and protective activity and is synthesized from L-arginine by the action of endothelial NO synthase (eNOS)[2]. Nitric oxide synthesized in endothelial cells diffuses to the smooth muscle cells of the vessel wall and activates guanilate cyclases, leading to vasodilatation. Shear stress is a key activator of eNOS. Endothelium also synthesizes other vasoactive substances such as cytochrome-derived factors, natriuretic peptide and prostacyclin; however, it appears that these substances have more limited roles in maintaining vasodilator activity than NO[3]. Nitric oxide also maintains vascular wall homeostasis by the inhibition of inflammation, cellular proliferation and thrombosis[4]. The imbalance between vasodilatory and vasoconstrictory substances incapacitates the vasodilatory response. These are the most recognized indicators of endothelial dysfunction.

## Endothelial Dysfunction and Atherosclerosis

Alteration in endothelial function is one of the earliest measurable markers of deterioration of the vessel wall in atherogenesis, which precedes the development of morphologic atherosclerotic changes and is a predictor of cardiovascular events[5, 6]. The involvement of endothelial dysfunction in atherogenesis is supported by findings which demonstrated that subjects with different risk factors of atherosclerosis, such as hypercholesterolemia, diabetes, hypertension and smoking, have significantly deteriorated endothelial function[7]. The progression of endothelial dysfunction is related to the intensity and duration of proven risk factors, and to the total risk of individual subjects[8, 9]. Endothelial dysfunction is a consequence of mechanical or chemical damage to the endothelium by different risk factors, and is closely related to cellular processes such as inflammation, which promotes the development of atherosclerosis. It was shown that C-reactive protein, one of the best recognized indicators of inflammation, induces the expression of adhesion molecules by human endothelial cells, which are markers of endothelial damage[10].

Endothelial dysfunction appears when the balance between NO production and its consumption is disrupted, and increased oxidative stress leads to unhealthy responses. Dysfunctional endothelium creates favorable conditions for platelet and leukocyte activation and adhesion, as well as the activation of cytokines that increase the permeability of vessel wall to oxidized lipoproteins and inflammation mediators, finally resulting in structural damage of the arterial wall with smooth muscle cell proliferation and plaque formation[11]. Endothelial dysfunction also plays an important role in the development and growth of atherosclerotic lesions, as well as in the appearance of ischemia and thrombosis in the late stages of the disease[12].

## Clinical Assessment of Endothelial Function

The development of clinical tests that evaluate the functional properties of normal and activated or damaged endothelium has improved understanding of the biology of the vascular endothelium. There are both invasive and non-invasive techniques for exploring various aspects of the pathobiology of the endothelium[1].

Most frequently, the endothelial function of the peripheral arteries is investigated; however, in the last decade invasive and non-invasive methods for the investigation of venous endothelium function were also developed.

## Assessment of Arterial Endothelial Function

Endothelial-dependent vasomotion has been the most widely used clinical end-point for assessment of endothelial function. Testing involves pharmacological and/or physiological stimulation of the endothelial release of NO and other vasoactive substances.

## Testing of Coronary Endothelial Function

Initial clinical studies of endothelial function were performed in the coronary circulation. This invasive technique involves local delivery of vasoactive agents (such as acetylcholine) with measurement of the changes in vessel diameter by quantitative coronary angiography[13]. The vasodilatory response of the investigated vessel can be also measured with the help of intravascular ultrasound. The vasodilatory agent delivered activates endothelial cells and stimulates NO release. Physiologically, in patients with normal endothelial response, the substance applied will release access to the vasodilator and result in dilation of blood vessels and hyperemia. In patients with endothelial dysfunction, this process is disturbed and the result is decreased vasodilation, thus demonstrating dysfunction; however, in certain patients, a paradoxical vasoconstriction event ensues[14]. Therefore, such a technique may carry the risk of coronary ischemia. Moreover, this technique is invasive and costly; therefore its

widespread use and clinical utility is limited.

## Flow-Mediated Dilation (FMD)

Recently, non-invasive methods for assessment of endothelial function were introduced. Exploration of the brachial artery flow-mediated dilation capability (FMD) is the most commonly used technique for investigation of endothelial function. Flow-mediated dilation serves as an index of NO-mediated endothelium-dependent vasodilator function. Endothelial dysfunction is reflected in an impaired FMD response[15, 16]. As endothelial dysfunction is a systemic process, and correlation between responses in the coronary circulation and in the forearm has been demonstrated[17], testing of the functional endothelium-dependent capability of peripheral arteries represents a window to investigation of the functional status of the whole circulatory system[10].

Investigation of vascular function is based on the technique described by Celermajer *et al.*[18]. To test the endothelium and smooth-muscle-dependent dilation capability, the brachial artery diameter proximal to the antecubital fossa is measured at rest and during reactive hyperemia. Measurements should be made at the end of diastole, which is determined by simultaneous monitoring of the electrocardiogram. Reactive hyperemia is achieved by rapid release of a pneumatic pressure cuff placed around the forearm and inflated to suprasystolic pressure for 5 min. The capability of endothelium-dependent (flow-mediated) vasodilation (a measure of endothelial dysfunction) is expressed as the change in the final diastolic diameter of the brachial artery during reactive hyperemia compared with its baseline value. A lack of vasodilation would suggest decreased release of endogenous vasodilators and therefore, endothelial dysfunction.

Different protocols have been introduced for measurement of the velocity and diameter of the artery under investigation. The standard and most frequently used technique is determination of the diameter of the investigated artery at a fixed time point (usually 60 seconds after cuff deflation); however, the use of a fixed time frame for FMD measurement has been recently discussed and questioned. Studies demonstrated that the arterial diameter observed at 60s underestimated the maximal FMD response[19]; therefore, continuous EKG-gated measurement of FMD starting with registration of the diameter before occlusion and then continuing during cuff inflation and for another 3-5 minutes after cuff deflation were suggested. Dyson *et al.* found that the FMD of the conduit peripheral arteries is dependent on the cumulative amplitude and duration of shear stimulus[20]. These investigations indicated that the use of continuous diameter measurement provides important data on endothelial function in healthy subjects and in patients with cardiovascular disease[21]. Furthermore, other indicative parameters of the FMD response have been proposed, including measurement of the time to peak dilation (time difference between the induction of reactive hyperemia and the peak dilatory response); however, it was shown that the time to maximal FMD does not appear to be a useful adjunctive measurement of endothelial function.

It was also shown that an occlusive cuff position influences FMD response and that the mechanisms of dilatory response differ. More proximal occlusion is accompanied by a significantly greater vasodilatory response[22].

Flow-mediated dilation of the brachial artery and the popliteal artery decreases with age, which may be attributable in part to decreased NO release and to diminished smooth muscle cell responsiveness in older subjects[23].

Since flow-mediated vasodilation is expressed as the change in post-stimulus diameter as a percentage of the baseline diameter, baseline diameter influences the change during stimulation. A larger baseline diameter yields a smaller percentage of change, and smaller arteries appear to dilate relatively more than larger arteries[24].

Flow-mediated dilation measurement has become popular in clinical studies because it strongly predicts cardiovascular events in patients with established cardiovascular disease. These studies generally indicated that FMD provides independent prognostic information, which may exceed the predictive value of traditional risk factors; therefore, FMD appears to be predictive of cardiovascular events in asymptomatic subjects and in those with established cardiovascular diseases. FMD is not only as predictive as traditional risk factors but may also provide important additional prognostic individual information on the risk of cardiovascular events[25]. Therefore, assessment of endothelial function on the basis of FMD has been proposed as a possible non-invasive and inexpensive endpoint that could reflect the cumulative cardiovascular burden and the responsiveness to therapies in individual patients[16, 26].

## Low-Flow-Mediated Constriction of Conduit Arteries

Flow-mediated dilation is a measure of vasodilation capability in response to a sudden increase in

shear stress and, as such, quantifies the capacity of the endothelium to cause smooth muscle cell relaxation and vasodilation when stimulated by a specific stimulus[27]. Despite FMD providing information on the capacity of the endothelium to increase its biosynthetic response (increase the bio-availability of NO), it does not measure resting endothelial activity, i.e. the endothelial production of vasomotor substances in resting conditions. To investigate vascular reactivity at rest, a new method was developed, low-flow-mediated constriction (L-FMC). Low-flow-mediated constriction quantifies the decrease in forearm conduit artery diameter that occurs in response to a decrease in blood flow and shear stress[28]. The measurement of L-FMC is usually combined with the determination of FMD, and a composite end-point of the absolute value of FMD and L-FMC is calculated. The methods employed for L-FMC/FMD determination differ in the investigation of different arterial segments, i.e. brachial vs. radial arteries. The technique of measuring L-FMC also differs in cuff position; most authors place the pneumatic cuff distal to the side of arterial diameter measurement[29], while others use the proximal cuff occlusion method[30]. Low-flow-mediated constriction is calculated as the percentage decrease in arterial diameter in the last 30s of cuff occlusion (after 4.5 min of 5 min occlusion) as compared with resting diameter[31]; however, FMD is determined in the 5 min following cuff deflation.

Low-flow-mediated constriction provides information concerning the control of arterial tone at rest, thus complementing and not overlapping the information provided by FMD. Studies also showed that there is no correlation between L-FMC and FMD[28]. These findings indicated that different mechanisms are responsible for basal and stimulated vasoactive response to shear stress. While the mechanisms underlying FMD are based on the synthesis and release of NO, inhibition of the synthesis of NO does not modify L-FMC, suggesting that NO has a less important role in maintaining the resting tone of arteries. The data show that under resting conditions, other substances such as prostaglandin are secreted by the endothelium of conduit arteries and that this production is decreased when shear stress is reduced[32]. Therefore, L-FMC might be a result of the common effect of vasodilator release (prostaglandins, endothelium-derived hyperpolarizing factor) and increased endothelin-1 production. It was also indicated that simultaneous measurement of FMD and L-FMC provides more comprehensive information on the different pathways involved in the control of vascular homeostasis.

In spite of the absence of an interrelationship between FMD and L-FMC, the results of both measurements demonstrated an association with traditional risk factors of atherosclerosis. Further, both L-FMC and FMD showed a dose-effect relationship with the severity of coronary artery disease[33]. These data demonstrate that assessment of different endothelial functions provide information that is additive and complementary to that of traditional risk factors[34].

## Peripheral Arterial Tonometry

Peripheral arterial tonometry (PAT) evaluates finger arterial pulse wave amplitude. PAT is a non-invasive technique that enables plethysmographic recording of pulse wave amplitude (PWA), which is a measure of pulsatile volume changes. Pulse wave amplitude is assessed before and during reactive hyperemia. The baseline PWA is determined using plethysmographic finger cuffs placed simultaneously on the index fingers of both hands for a period of 5 minutes. Hyperemia is induced by occluding blood flow through the brachial artery for 5 minutes using an inflatable cuff. The PWA reactive hyperemia index is calculated as the ratio of the average PWA between post- and preocclusion values. These values are normalized to measurements of the contralateral arm, which serves as a control of the non-endothelial-dependent systemic effects of reactive hyperemia[35, 36].

The physiological mechanisms underlying the PWA response are not completely understood. Although some studies have demonstrated a direct contribution of nitric oxide to both the brachial artery FMD and the digital PAT values[37], conflicting results regarding the correlation between these two investigative methods have been reported[35, 38, 39]. No correlation was observed in the Guttenberg Heart Study[39]. In the study of Lee *et al.*, no correlation was observed between PWA and FMD during reactive hyperemia. On the other hand, this study was the first to report the presence of a significant positive relation between digital PWA and baseline brachial artery blood flow velocity. Although increases in peripheral artery diameter after reactive hyperemia are mostly dependent on nitric oxide bio-availability, changes of blood flow are widely recognized as an index of micro-vascular, but not macro-vascular, function[40]. Studies have also demonstrated that as registered by PAT, vascular dysfunction is associated with risk factors and the presence of cardiovascular disease[24, 41]. These findings suggest that the digital PWA ratio and brachial artery FMD assess distinct vascular functions.

Abnormalities of the pulse wave amplitude have long been described in the peripheral circulation of

patients with atherosclerosis[42]. Furthermore, PAT hyperemia has been shown to be an adequate surrogate marker to access changes in vascular function over time, and in the Framingham cohort it was closely linked with cardiovascular risk factors[35]. An abnormal vascular response registered by PAT was also shown to be prevalent in adolescents with type 1 diabetes mellitus[43]. The study from Kitta *et al*. showed that PWA predicts future adverse outcome events in patients with obstructive coronary artery disease[44]. Studies also indicated a relationship between coronary endothelial dysfunction, as detected by invasive evaluation of the coronary arteries and the findings of PAT[45]; therefore, PAT potentially offers a more simplified non-invasive measurement, although it measures vascular functions other than FMD.

## Determination of Venous Endothelial Dysfunction

As the endothelium is one of the components that regulate the functional capability of the whole circulatory system, it is expected that it regulates the functional status not only in the arteries but also in the venous system. In the veins, the endothelium maintains circulatory homeostasis through its control of vasomotion, coagulation, fibrinolysis and platelet activation. Investigations of the pathogenesis of venous and arterial thrombosis show similarities, particularly in the involvement of the endothelium. Endothelial dysfunction or damage to endothelial cells may represent the common pathogenetic background of venous thrombembolism and arterial thrombosis. Deteriorated vessel wall function (venous or arterial) could promote thrombus formation. The link between endothelial dysfunction and venous thrombosis has been established in several studies[46] and, interestingly, endothelial microparticles, which have been shown to regulate venous tone, were detected in patients with venous thrombosis[47, 48].

Since more than 70% of the total circulating blood volume is contained in the venous vascular bed, it is conceivable that small changes in venous tone may substantially affect filling pressure, making the evaluation of venous endothelial function an attractive new method to evaluate vascular homeostasis.

In contrast to evaluation of the endothelial function of the peripheral arteries, the determination of venous endothelial function is more complicated. For assessment of endothelial function, different invasive and non-invasive techniques are used, most frequently, a minimally invasive method known as the "dorsal hand vein technique"[49]. This method evaluates and quantifies the vascular responsiveness of the pre-constricted dorsal hand vein to different substances (acetylcholine, sodium nitroprusside, norepinephrine). In a suitable vein on the dorsum of the hand, a needle is inserted into which a linear transformer is mounted, providing measurement of the vein diameter. The readings are usually made under congestive venous pressure produced by inflating a pressure cuff placed on the upper portion of the arm[50]. This technique is minimally invasive, but because of the influence of numerous factors, the measurement has poor reproducibility.

It is also possible to assess venous tone in the forearm capacitance bed by radionuclide venous plethysmography[51]. This technique involves labeling red blood cells with technetium-99m ($^{99m}$Tc). The radioactive counts registered are proportional to forearm blood volume. Determination of the venous volume/pressure relation allows assessment of venous tone or venous compliance.

All techniques for direct determination of venous endothelial function are complicated and their reproducibility is limited; therefore, measurement of endothelial function of the peripheral arteries is frequently used as a surrogate to assess venous endothelial function[52, 53]. Namely, in subjects with deteriorated arterial endothelial function, venous endothelial dysfunction is also expected.

## Applicability and Utility of Different Methods for Investigation of Endothelial Function

Endothelial dysfunction is a key underlying factor in the atherosclerotic process and the earliest measurable functional abnormality of the vessel wall in atherogenesis. Therefore, different tests of endothelial function have been sought, particularly those involving disturbed endothelium-dependent vasomotion. All available methods aim to evaluate endothelial function/dysfunction; however, they differ in investigating the mechanisms of endothelial dysfunction. Further, different techniques investigate the functional capability of various sections of the circulatory system (**Table 1**). Only some of these methods have been standardized and accepted for routine clinical use.

Endothelial-dependent flow-mediated dilation of conduit arteries was one of the first non-invasive methods for clinical assessment of endothelial function, and its validity and applicability were defined in consensus documents[26]. It is an indicator of the vessel wall dilation capability of large conduit arteries. Studies have confirmed the close interrelationship between FMD and total cardiovascular risk and the extent of

**Table 1.** Validity and differences between various methods for investigation of endothelial function/dysfunction

| TECHNIQUE | TARGET OF INVESTIGATION | UTILITY |
|---|---|---|
| | ARTERIAL SYSTEM | |
| FMD | Large, conduit arteries (peripheral, coronary) | Determination of endothelial function of large arteries and its relationship to risk factors of atherosclerosis and atherosclerotic disease |
| L-FMC | Peripheral-conduit arteries | Measurement of endothelial activity at rest during decreased blood flow and shear stress. Provides information on different pathways involved in regulation of vascular tonus |
| PAT | Small (digital) arteries and microcirculation | Registration of changes in blood flow and calculation of microvascular function |
| | VENOUS SYSTEM | |
| DORSAL HAND VEIN TECHNIQUE | Dorsal hand vein | Evaluation and quantification of functional responsiveness of peripheral veins |
| RADIONUCLIDE VENOUS PLETHYSMOGRAPHY | Venous forearm capacitance veins | Registration of forearm blood flow and assessment of venous tone or venous compliance |

FMD: flow-mediated dilation, L-FMC: low-flow-mediated constriction, PAT: peripheral arterial tonometry

atherosclerosis[54]. Flow-mediated dilation process is based on NO release from endothelial cells; therefore, blunted dilation is expected in subjects with increased oxidative stress as the consequence of the presence of risk factors of atherosclerosis.

Low-flow-mediated constriction of conduit arteries quantifies the decrease in forearm conduit artery diameter that occurs in response to decreased blood flow and shear stress. This method is not predominantly based on NO availability but it might be mediated by other substances and is probably a common effect of the release of different vasodilators: prostaglandins and endothelium-derived hyperpolarizing factor. Therefore, L-FMC provides different information than FMD and is used as a complementary technique for measurement of endothelium-dependent flow-mediated dilation. The combination of both techniques provides more comprehensive information on the different pathways involved in the control of vascular tonus.

Peripheral arterial tonometry is based on plethysmographic recording of the pulse wave amplitude. The physiological mechanisms underlying PWA response are not completely understood. Studies indicated that, as in FMD, NO plays an important role in digital PAT[37]. Changes in blood flow registered by PAT are indicators of microvascular but not macrovascular function; therefore, digital PAT assesses vascular function distinct to that investigated by FMD. According to this presumption, PAT would be useful for the determination of endothelial dysfunction at the level of microcirculation and resistance arteries in diseases accompanied by microangiopathy; however, the utility and value of PAT measurement are less established than FMD.

Techniques for the evaluation of venous endothelial function are more complicated and much less proven than methods for the assessment of arterial endothelial function. The reproducibility of both techniques for studying venous endothelial function (direct measurement of vascular responsiveness of dorsal hand vein and radionuclide plethysmography) is poor and their validity should be confirmed in large clinical series; however, as it is expected that endothelial dysfunction is a systemic disorder, measurement of arterial endothelial function can be used as a surrogate for the assessment of venous endothelial function.

## Conflict of Interest

None.

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