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# Methods for evaluating endothelial function: a position statement from the European Society of Cardiology Working Group on Peripheral Circulation.



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# METHODS FOR EVALUATING ENDOTHELIAL FUNCTION. A POSITION STATEMENT FROM THE EUROPEAN SOCIETY OF CARDIOLOGY WORKING GROUP ON PERIPHERAL CIRCULATION.

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## ABSTRACT

The endothelium holds a pivotal role in cardiovascular health and disease. Assessment of its function was until recently limited to experimental designs due to its location. The advent of novel techniques has facilitated testing on a more detailed basis, with focus on distinct pathways. This review presents available in-vivo and ex-vivo methods for evaluating endothelial function with special focus on more recent ones. The diagnostic modalities covered include assessment of epicardial and microvascular coronary endothelial function, local vasodilation by venous occlusion plethysmography and flow-mediated dilatation, arterial pulse wave analysis and pulse amplitude tonometry, microvascular blood flow by laser Doppler flowmetry, biochemical markers and bioassays, measurement of endothelial-derived microparticles and progenitor cells, and glycocalyx measurements. Insights and practical information on the theoretical basis, methodological aspects, and clinical application in various disease states are discussed. The ability of these methods to detect endothelial dysfunction before overt cardiovascular disease manifests make them attractive clinical tools for prevention and rehabilitation.



**Keywords:** biomarkers; cell-derived microparticles; coronary angiography; endothelium; glycocalyx; nitric oxide; plethysmography; progenitor cells; pulse; laser-doppler flowmetry

## Endothelial function: key biochemical concepts

The endothelium actively regulates basal vascular tone and reactivity under both physiological and pathological conditions; its activity extends far beyond the control of vascular tone and vasomotion. In addition, a functional endothelium is a major regulator of vascular inflammation and remodelling. An intact endothelial layer is critical for preventing circulating blood cells from exposure to prothrombotic subendothelial matrix and inhibits arterial thrombus formation by limiting platelet activation, adhesion, and aggregation. In the presence of cardiovascular risk factors, an excessive production of superoxide ($O2^{-}$) occurs rapidly, inactivating nitric oxide (NO). Loss of NO bioavailability precedes the development of overt atherosclerosis and is an independent predictor of adverse cardiovascular events. Efforts have been made at translating this knowledge into clinical applications by developing methods for assessing endothelial function in coronary arteries. Subsequently, these applications have been expanded to other vascular territories.

## Coronary endothelial function

Despite its invasive nature, angiography remains a broadly used technique for assessing endothelial function of epicardial coronary arteries owing to its availability in clinical routine. Pharmacological stimuli to assess epicardial coronary vasodilation include intracoronary infusion of acetylcholine (ACh), metacholine, or papaverine. ACh has been widely used due to the short duration of its effect and favourable safety features. It typically causes paradoxical vasoconstriction, as opposed to dilatation, in endothelium-denuded arteries in vitro as well as in atherosclerotic coronary arteries in vivo, in part due to impaired muscarinic cholinergic vasodilation in coronary atherosclero-

sis.1,2 Adenosine is an endothelium-independent vasodilator primarily of the microcirculation. It therefore induces an increase in blood flow, which can be used to assess flow-mediated vasodilatation. Nitroglycerin has been routinely used to assess endothelium-independent vasodilation owing to direct relaxation of vascular smooth muscle cells. It is important to administer intracoronary vasodilator agents as infusions of increasing concentrations to assess dose-response effects.

Methodology

For epicardial coronary vessels, quantitative coronary angiography (QCA) measures changes in their diameter. Changes are usually compared with baseline conditions and with vasodilation induced by endotheliumindependent drugs. More recently, non-invasive QCA has been developed by using computed tomography imaging3 or magnetic resonance imaging (MRI).4 In the microvasculature, changes in coronary blood flow are assessed by vessel diameter and Doppler flow velocities (online supplemental material and Supplemental Figure 1).5 Given the invasive nature of the technique, patients should be selected carefully. As an example, patients presenting with left main coronary artery stenosis should not be considered for this procedure. Non-invasive methods, such as MRI and positron emission tomography (PET), for assessment of coronary microvascular function have shown impressive improvements. MRI allows an accurate quantification of myocardial function, infarct size, and microvascular function. PET imaging is a method of choice for myocardial blow flow measurement. Combined with pharmacological or physiological endotheliumdependent stimuli (e.g. cold pressor testing), it is very valuable in assessing microvascular coronary endothelial function.6

An additional method that holds great promise for non-invasive assessment of microvascular function is thermodilution.[7,8]

Clinical applications

Epicardial and microvascular coronary endothelial dysfunction is an independent predictor of acute cardiovascular events irrespective of presence or absence of angiographically detectable coronary lesions.[9,10] Assessment of coronary endothelial function is particularly important in cardiac transplant recipients because the endothelium is an early target of ischaemic, immunological, and pharmacological graft injury, including ciclosporin-related vasculopathy. In addition, the concentric nature (intimal thickening) and diffuse distribution of graft coronary artery lesions may hamper their early detection by angiography. Both epicardial and microvascular endothelium-dependent vasomotor dysfunction has been shown to correlate with the development of graft atherosclerosis in transplanted patients.[11]

**Venous occlusion plethysmography**

Venous occlusion plethysmography (VOP), established more than 100 years ago, is the longest living method for investigating blood flow in humans.[12]

Methodology

The main principle of VOP is based on the measurement of tissue (usually muscular) blood flow by the assessment of the tissue volume change, which is induced by the inflation of a cuff proximally to the under evaluation tissue. The cuff is inflated up to that pressure which occludes venous outflow (return) but allows arterial inflow. The rate

of the volume change is therefore proportional to the rate of arterial inflow. She most widely applied method of VOP is based on the use of automatically calibrated mercury-in-silastic strain gauges,[13,14] which are placed as rings around the under examination limb. A minimally invasive, modified strain-gauge VOP method is being applied in the forearm since the early 1990s in order to investigate in vivo the endothelial function in the human microcirculation. This technique requires brachial artery cannulation for intra-arterial infusion of endothelial agonists (mainlyACh or metacholine, bradykinin, etc.), which act via specific receptors and induceNOproduction fromendothelial cells. Endothelial agonists are infused in escalating doses that induce local vasodilation without systemic haemodynamic effects.[15,16] Endothelial function is estimated by the dose–esponse of the forearm blood flow (FBF, ml/min/100ml tissue) due to the local endothelium-dependent vasodilation. Subsequent intra-arterial infusion of direct smooth muscle relaxing factors (e.g. nitrates) is used in order to evaluate the effect of endothelium-independent vasodilation on FBF. Ideally, simultaneous recordings of FBF at the contralateral arm are required in order to verify the absence of any systemic haemodynamic effects, induced by the intra-arterial drug infusion. Strain-gauge VOP is a highly reproducible method that provides valid results with the investigation of a relatively limited sample size. Moreover, due to its invasive nature, it permits the local infusion of substances enabling the investigation of endothelial function. Therefore it is an excellent research tool. On the other hand, the main disadvantage of the FBF technique is its very same invasive nature, which limits its application in clinical practice.

Clinical applications

A very large body of evidence documented the presence of impaired endothelium-dependent relaxation (low ACh-induced FBF, ACh-FBF) microcirculation in patients with cardiovascular risk factors.[15,16] Moreover, prospective studies have shown that endothelial dysfunction, as assessed by ACh-FBF, is an independent predictor of cardiovascular events in subjects with essential hypertension[17] and coronary artery disease.[18,19]

**Flow-mediated dilatation**

Conduit vessels respond to alterations in blood flow by increasing vessel diameter via an endothelial dependent mechanism.[20,21] The flow-mediated dilatation (FMD) technique measures changes in conduit artery diameter by ultrasound. This response has been shown to reflect local bioactivity of endothelial-derived NO.[22]

Methodology

The brachial artery is most often imaged (online Supplemental Figure 2). FMD studies are performed in a quiet temperature controlled room while subjects are lying supine for >10 min prior to image acquisition. A straight, non-branching segment of the brachial artery above the antecubital fossa is imaged in the longitudinal plane with the ultrasound probe securely fixed using a stereotactic clamp. This permits fine adjustments in the coronal and sagittal planes. A blood pressure cuff is placed 1-2 cm below the antecubital fossa and inflated to supra-systolic pressure. [23] After cuff release, reactive hyperaemia results and is quantified using Doppler. The arterial diameter is recorded at end diastole using electrocardiographic gating during image acquisition, to determine the response of the conduit artery to increase in flow.[24] Changes in the arterial diameter are assessed using commercial digital edge detection software. Flow-mediated dilatation

is expressed as a percentage change of the arterial diameter from the baseline vessel size. Phantom studies have demonstrated that differences in absolute diameter of as little as 0.04mm can be detected and differences in artery diameter of 0.1mm can be reliably distinguished.[25] Inter- and intraobserver variability studies and assessment of change in FMD over time have enabled construction of power curves for different clinical trial protocols.[26] Well-trained operators are essential to obtain such accurate, reproducible measurements.

Clinical applications

FMD has been shown to be impaired in response to a range of classical and novel risk factors from the first decade of life and to decline in proportion to risk factor burden.[27] In addition, it was shown that impaired endothelial function is associated with more rapid progression of a measure of structural arterial disease, carotid intima media thickness in an asymptomatic middleaged population.[28] Several studies have shown a relationship between endothelial dysfunction and clinical events in high-risk cohorts.[9,29] FMD changes rapidly and can be restored by beneficial lifestyle changes[30] and medical therapy such as statin use.[31]

**Pulse wave analysis**

The arterial waveform contains important information about the stiffness of the large arteries and amount of wave reflection within the arterial system.[32] Wave reflection occurs at sites of impedance mismatch, often branch points, and is usually quantified by determining the augmentation index (AIx), which represents the difference between the first and second systolic peaks.[33] Although the impedance of the large, elastic

arteries is relatively static, impedance of the small arteries and arterioles is much more dynamic and depends to a large extent on smooth muscle tone and vessel size. Thus, changes in small artery tone affect wave reflection: vasodilatation reduces AIx, whereas vasoconstriction increases it.[33] One of the main factors regulating vascular tone is endothelium-derived NO, and it is now clear that, through this mechanism, NO also influences wave reflection and, therefore, the shape of the arterial wave. Glyceryl trinitrate (GTN), an NO donor, reduces wave reflection at low doses before any measurable effect on resistance or mean pressure,[34] suggesting that small arteries are more sensitive to GTN than resistance vessels. Conversely, inhibiting endogenous NO production with LG-monomethyl L-arginine increases wave reflection.[35] Klemsdal et al.[36] demonstrated that ACh, an endothelium-dependent nitrovasodilator, reduces wave reflection in rabbits and that this effect is attenuated in hypercholesterolaemic animals with endothelial dysfunction. This important observation led Chowienczyk et al.[37] to propose that endothelial function could be assessed in humans by recording the shape of the arterial waveform and administering GTN as an endothelium-independent stimulus and the endothelium-dependent agonist salbutamol. Since both drugs could be given non-invasively – sublingual and inhaled, respectively – this meant a truly non-invasive method for assessing endothelial function. Subsequent work confirmed the endotheliumdependent nature of salbutamol's effect on AIx and that the technique was repeatable and correlated with more established methodologies.[38]

Methodology

A number of versions have been described, but all rely on non-invasive waveform recording, and administration of GTN and a b2 agonist. Details on the procedure can be found in online supplemental material and Supplemental Figure 3.

Clinical applications

Given the novelty of the technique, relatively few clinical studies have employed pulse wave analysis (PWA) to assess endothelial function. However, impaired endothelium-dependent responses have been observed in conditions traditionally associated with endothelial dysfunction, including diabetes,[37] hypercholesterolaemia, [38] coronary artery disease,[39] peripheral vascular disease,[40] and rheumatoid arthritis.41 The GTN response is usually preserved. An inverse correlation between terbutaline response and Framingham risk score has been reported,[42] along with a stronger correlation between individual risk factors and endothelial function assessed with PWA compared to FMD. At present there are no data relating the PWA technique to treatment effects or outcome.

**Peripheral arterial tonometry**

Measurement of peripheral vasodilator response with fingertip peripheral arterial tonometry (PAT) technology (EndoPAT; ItamarMedical, Caesarea, Israel) is emerging as a useful method for assessing vascular function.[43] Although PAT signal is modulated by various local, systemic, and environmental factors, this parameter is also affected by the bioavailability of NO and, therefore, also depends on endothelial function.[44] In response to hyperaemic flow, digital pulse amplitude (and hence PATsignal amplitude) increases, a response that has been shown to depend in part on NO synthesis.[45]

Methodology

The EndoPAT device consists of two finger-mounted probes, which include a system of inflatable neoprene membranes within a rigid external case. A blood pressure cuff is placed on one upper arm (study arm), while the contralateral arm serves as a control (control arm). The EndoScore is calculated as the ratio of the average amplitude of the PAT signal over a 1-min time interval starting 1.5 min after cuff deflation divided by the average amplitude of the PAT signal of a 3.5-min time period before cuff inflation (baseline) (Figure 1).[46] Augmentation index is also automatically calculated as an average from multiple pulses using the formula (P2-P1)/P1 (%) where P1 is the peak pressure of the recorded pulse wave and P2 is the pressure of the inflection point corresponding to the arrival of the reflected waves.

Clinical applications

Digital reactive hyperaemia, as measured by EndoScore, is attenuated in patients with coronary endothelial dysfunction, suggesting a role as a non-invasive tool to identify patients during the early stage of CAD.[46] The EndoScore was reduced in subjects with impaired endothelial function represented by low FMD and was higher in subjects with greater brachial artery FMD responses consistent with more preserved endothelial function.[43] The EndoScore was more impaired in subjects with exercise myocardial perfusion imaging studies that were indicative of coronary artery disease.[43] In participants of the Framingham Heart Study, obesity and diabetes mellitus, along with the associated dyslipidaemia and insulin resistance, have been linked to impaired vasodilator responses.[47,48] Measurements of the PAT index in healthy male adults demonstrated sig-

nificantly increased responses after the ingestion of a high-flavanol cocoa drink as compared with after a low-flavanol cocoa drink.[49] Regarding the incremental value of PAT, patients at low Framingham Risk Score risk but with endothelial dysfunction are at a higher actual risk than patients with high Framingham Risk Score but normal endothelial function. Furthermore, endothelial dysfunction was found to be an independent risk factor for a future major adverse cardiovascular event.[50] Research work is lacking for the evaluation of the potential role of EndoScore in risk stratification for individuals. Data on circadian variation, changes after meals and during concurrent illnesses, or after cardioactive medication, blood pressure lowering and/or lipid lowering interventions are also missing.

**Laser Doppler flowmetry**

Laser Doppler flowmetry (LDF) is a technique enabling the monitoring of skin microvascular blood flow.[51] The assumption is that the response observed in the cutaneous circulation is a window towards the responses that should be observed in other vascular beds.[52]

Methodology

During LDF, the original beam of coherent light changes in contact with moving tissues (red blood cells) and a photodiode measures the emerged beam. The fraction of shifted light depends on the concentration of moving red blood cells, whereas the magnitude of the frequency broadening depends on their average velocity.[53] Many techniques can be associated to LDF to estimate endothelial function among which direct delivery of ACh through iontophoresis or micro-dialysis (Figure 2). Other techniques

can also be used such as post-occlusive hyperaemia or local skin heating. The major advantages are that LDF measures skin blood flow in a non-invasive manner and the direct delivery of ACh to the tissues. The response is monitored non-invasively when using iontophoresis or with the minimally invasive micro-dialysis technique. Microdialysis requires that a small micro-porous catheter is inserted under the epidermal surface. Iontophoresis, the migration of charged substances through the skin by means of small continuous galvanic current, allows very small amounts of drugs to be administered non-invasively and safely to a localized area.[54] The ACh induced vasodilatation during iontophoresis is biphasic and involves prostanoid participation.[55] Various other drugs (adrenaline, insuline, sodium nitroprusside, etc.) can be used to test different pathways. These have almost no systemic effect, allowing the use of the technique even in neonates.[56] Further details on LDF methodology can be found in the online supplemental material.

Clinical applications

ACh iontophoresis is impaired in disease states such as sleep obstructive apnoea syndrome,[57] obesity or diabetes, [58,59] heart transplant recipients,[60] hypercholesterolaemia or hypertension61 even in the absence of macrovascular lesions, and the impaired response can be improved after treatment.[62] To date, an altered response to ACh iontophoresis cannot be used for treatment indication, although it has been shown that many drugs that have proved benefit in cardiovascular disease or diabetes can improve the ACh response.[62–64] Spectral analysis of the LDF signal, standardization of the protocols, and a better knowledge of technical issues of iontophoresis are still needed. Nevertheless, LDF is an easy and relatively cost-effective technique to approach endothelial function;

furthermore, it offers the unique opportunity to test various pathways of the vascular response.

**Biochemical markers and bioassays**

Several markers have been used to examine different aspects of endothelial cell functions (Figure 3). This review focuses on asymmetrical dimethylarginine (ADMA) and oxidized low-density lipoprotein (oxLDL), two key markers of endothelial function. ADMA, a product of arginine methylation, represents an endogenous inhibitor of endothelial NO synthase.[65] ADMA is, however, not a specific endothelial NO synthase inhibitor, but does also inhibit the other NO synthases (iNOS and nNOS). Cross-sectional studies have suggested that ADMA plasma concentrations are increased in patients with vascular disease, renal failure, or cardiovascular risk factors.[66] A large recent cross-sectional analysis suggested an overall significant, but rather modest inverse association between ADMA and endothelial function as measured by flow-dependent vasodilation.[67] Studies suggest that increased ADMA plasma levels are independently associated with an elevated risk of future cardiovascular events;[68,69] however, a recent analysis did not observe an association of ADMA plasma levels with the incidence of cardiovascular disease.[70] Therefore, at present, ADMA measurements may not represent a reliable 'replacement' of a direct endothelial function measurement, and its association with cardiovascular prognosis may depend on the patient cohort studied. Oxidized LDL has been suggested to contribute importantly to endothelial dysfunction and progression of atherosclerosis. Endothelial activation in response to oxidized LDL is likely in particular mediated by the lectin-like oxidized LDL receptor (LOX-1) present on endothelial cells. Oxidized LDL levels are difficult to assess in vivo, and in plasma there are likely

only minimally oxidized LDLs present. Several monoclonal antibodies have been used to characterize circulating oxidized LDL levels.[71] Increased levels of oxidized LDL were predictive of future coronary disease events in apparently healthy men,[72] while plasma oxidized LDL levels predicted the risk of CHD in a large prospective study.[73] However, after adjustment for lipid markers such as apoB and LDL, no independent predictive value for plasma oxidized LDL levels was observed. Endothelial bioassays can be employed in order to examine the potential effects of therapeutic interventions on endothelial cell functions. Isolated highdensity lipoprotein (HDL) from healthy subjects stimulates endothelial cell NO production, whereas HDL isolated from diabetic patients with low HDL failed to do so,[74] suggesting that the endothelial effects of HDL are markedly altered in these patients and can be partly restored by niacin therapy. In vitro, pathological levels of oxidized LDL are directly cytotoxic to human umbilical vein endothelial cells, and this results in increased release of von Willebrand factor, which is associated with a risk of future coronary heart disease.[75]

**Endothelial microparticles**

Microparticles (MPs) are vesicles (100nm to 1 mm diameter) shed from plasma membranes following cell activation or apoptosis.[76] MPs of different cellular origins (platelets, leukocytes, red blood cells, endothelial cells, etc.) are found in the plasma of healthy subjects and their circulating levels augment in atherothrombotic diseases. Although MPs originating from endothelial cells (EMP) represent a small fraction of the overall pool of plasma MPs, increased circulating EMP levels constitute an emerging surrogate marker of endothelial dysfunction. If the mechanisms leading to their in-vivo formation remain obscure, release of EMPs from cultured cells is increased by tumor

necrosis factor a, thrombin, uraemic toxins, and reactive oxygen species. Endothelial apoptosis does not appear to be a prerequisite for EMPs generation.[77] Interestingly, endothelial NOS uncoupling contributes to EMP release, emphasizing the possible reciprocal relationships between EMP and endothelial dysfunction.[78] Low shear stress associates with increased EMP levels,[79] suggesting that physiological shear stress, known to contribute to endothelial survival, may limit EMPs release.

Methodology

Microparticles can be numbered and their cellular origin characterized using different methodological approaches based on the identification of cell-surface clusters of differentiation (CD) using specific antibodies and/or the presence of externalized phosphatidylserine, which does not discriminate between MP of different cellular origin. Great care should be taken to use appropriate controls (e.g. no calcium for annexin V labelling) and appropriate isotypes to assess unspecific labelling. EMP may express adhesion molecules harboured by mature endothelial cells such as CD54 (ICAM-1), CD106 (VCAM-1), CD62E (E-selectin), CD62P (P-selectin), or CD31 (PECAM-1). EMP also may harbor CD105 (endoglin, also expressed by activated monocyte-macrophages and bone marrow cell subsets), CD144 (VE-cadherin), and CD146 (S endo-1, an endothelial junctional protein), and they bind von Willebrand factor. Proteomic studies report different EMP phenotypes depending of the vesiculation stimulus. So far, CD144, CD146, and CD62E appear to be the most specific markers attesting for the endothelial origin of MPs (Figure 4). Methodological details regarding EMP assessment can be found in the online supplemental material.

Clinical applications

Circulating EMP levels increase in several cardiovascular and atherothrombotic diseases.[80] In end-stage renal disease, EMP plasma levels correlate with arterial stiffness and the impairment of flow-mediated dilatation independently of age and blood pressure, whereas such relationship was not observed for other MP populations.[81] Similar results have been found in diabetes[82] and obesity.[83] In acute coronary syndromes, increased circulating levels of EMPs correlate with the extent of endothelial dysfunction.[84] EMPs appear as a more robust predictor of the occurrence of myocardial infarction in diabetic patients, when compared to classical markers of endothelial activation.[85] In addition, plasma EMP levels correlate positively with the extent and severity of coronary stenosis at angiography in patients with coronary syndromes.[86] Plasma EMP levels are also increased in patients with pulmonary hypertension where they predict the severity of the disease.[87] Increased EMP plasma levels not only reflect endothelial activation or apoptosis, but they may also increase endothelial dysfunction by impairing endothelium-dependent dilation and the endothelial NO pathway.[88]

## Endothelial progenitor cells

Cardiovascular risk factors damage the endothelial monolayer by inducing endothelial cell senescence and apoptosis, leading to endothelial dysfunction. The restoration and reconstitution of the damaged endothelial monolayer may be a prerequisite for the prevention of endothelial dysfunction and atherosclerotic lesion formation. Until recently endothelial cell repair mechanisms were thought to be mediated by the adjacent endothelial cells. However, adult blood vessels regenerate only moderately in adults under physiological conditions. The half-life of an adult endothelial cell has been reported to

be 3.1 years.[89] A growing body of evidence suggest that circulating, bone marrowderived, endothelial progenitor cells (EPC) play an important role in endothelial cell regeneration.[90] EPC can be measured from peripheral blood either by direct enumeration using flow cytometry (CD34/KDR, CD34/KDR/CD133 positive EPC) or after cultivation of mononuclear cells (early and late outgrowth EPC).[91,92] Methodology Flow cytometry. Flow cytometry is used to measure circulating EPC within peripheral blood. Most publications use CD34, CD133, and KDR (VEGFR2) as surface markers.[91,93] The advantages of this technique include its ability to enumerate untouched, naı¨ve cells with a defined phenotype and the identification of specific cell populations in multicolour fluorescence-activated cell sorting analysis. On the other hand, the definition of cells based on surface markers is under heavy debate, while surface markers do not necessarily correlate with cell function. In-vitro assays. In-vitro assays have been widely used to identify EPC.[94] Two major cell populations have been recently described: early and late outgrowth EPC.[92] Early outgrowth EPCs are derived from mononuclear cells and are cultured using EPC differentiation medium. Regarding late outgrowth EPC, there is currently no data available for their role in endothelial function. Reproducibility and repeatability severely depend on the method used. Cell enumeration of rare cells using fluorescence-activated cell sorting analysis is highly dependent on the protocol used and the experience of investigator. A standardized protocol with clinical evaluation has been published.[91] Further details on standardization issues can be found in the online supplemental material.

Clinical applications

Various publications have demonstrated a correlation between EPC, severity of endothelial dysfunction measured either invasively or non-invasively, and outcomes.[93,95,96] However, others[97,98] have failed to describe a correlation between EPC and endothelial function. All published results are descriptive and no clinical trials are available demonstrating that therapeutic interventions of circulating EPC numbers can improve endothelial function. First hints that stem and progenitor cells may positively influence impaired endothelial function comes from a substudy of the REPAIR-AMI trial demonstrating restoration of microvascular function of the infarct-related artery after bone marrow stem cell transfusion.[99] Mobilization therapy using drugs and substances with effects on the number of circulating EPC has shown an improvement of endothelial function.[100] A clear causal role has not been established yet. Currently, there is no strong evidence for a clear dependency of EPC numbers (within circulating blood or subcultured EPC) with the extent of endothelial dysfunction. Additionally, the role of EPC as a guide to treatment has not been established yet. In the clinical setting, more and larger clinical studies evaluating EPC and its correlation with endothelial function are needed. Furthermore, large clinical trials are warranted with an interventional approach to enhance the number of circulating EPC in order to establish the role of EPC in improving endothelial function.

**Endothelial glycocalyx**

The glycocalyx forms a gel-like layer covering the endothelial lining, shielding endothelial cells from direct contact with circulating blood cells (Figure 5). This strategic position already implies its potential role in several crucial balances at the level of the

vessel wall, including the vasoconstrictor–vasodilator, the pro- and anticoagulant, the pro- and anti-inflammatory, as well as the pro- and antioxidative balance. Under physiological conditions, the endothelial glycocalyx preserves the integrity of the vessel wall. It serves as an inert barrier that precludes direct 'endothelial' contact and creates a selectively permeable structure contributing to the generation of the osmotic pressure gradient across the vessel wall. The glycocalyx serves as an active reservoir containing major enzymatic systems and their cofactors [lipoprotein lipase (LPL), extracellular superoxide dismutase (ec-SOD), antithrombin III (AT III), anticoagulant heparansulphates and thrombomodulin].[101] It has an important role as mechano-transductor, transferring shear stress into shear-dependent endothelial responses; this leads to NO release.[102] Damage to the endothelial glycocalyx is associated with influx of lipoproteins, leakage of macromolecules, and adhesion of circulating cells to the endothelium. Concomitantly, loss of glycocalyx may contribute to an imbalance in enzymatic systems such as coagulation and antioxidant defence as well as an impaired NO release.[103] As glycosaminoglycan (GAG) synthesis is under direct negative feedback of inflammatory and oxidative stimuli,[104] this indicates that a pro-atherogenic environment has a direct impact on both GAG composition as well as glycocalyx dimension. In turn, both a decreased rate and aberrant composition of GAG synthesis may contribute to vascular inflammation and vascular dysfunction.

Methodology

Glycocalyx measurements can be performed using invasive or non-invasive techniques. In the first technique, systemic glycocalyx volume is measured using the glycocalyx permeable tracer Dextran 40 versus a glycocalyx impermeable tracer, fluorescein-

labelled erythrocytes. The calculated difference between the two intravascular volumes that these tracers occupy renders a reasonable estimation of whole-body glycocalyx volume.[105] However, the invasive nature and the timeconsuming preparations precludes its use in larger cohorts. Moreover, it is not clear whether this method considers the endothelial glycocalyx as uniformly distributed throughout the vasculature. Nevertheless, this technique may be of use in order to obtain a first indication of systemic glycocalyx dimensions in humans. The second, non-invasive technique uses a semiautomated imaging method to estimate glycocalyx thickness by OPS (Orthogonal Polarization Spectral imaging) or SDF (Sideview Darkfield imaging) at the superficial microvasculature. Glycocalyx limits the proximity of erythrocytes to the capillary endothelial cells; this imaging method uses the erythrocyte– endothelium gap of the capillaries in the image to quantify glycocalyx thickness.

Clinical applications

Controlled inflammatory challenges lead to instantaneous shedding of glycocalyx constituents and leukocyte activation in conjunction with a profound decrease in glycocalyx dimension. Pre-treatment with etanercept, a soluble tumour necrosis factor a receptor, prevented leukocyte activation as well as shedding of glycocalyx compounds.[106] Acute hyperglycaemia results in a perturbation of the endothelial glycocalyx with a concomitant increase in vascular permeability,[105,107] and changes in glycocalyx biochemistry are associated with carotid intima media thickness, an established marker of cardiovascular disease, in type 1 diabetes mellitus patients.[107] In patients with familial hypercholesterolemia, an inverse relationship between plasma LDL cholesterol levels

and glycocalyx dimensions exists, whereas cholesterol- lowering treatment resulted in partial recovery of glycocalyx.108

## Perspectives

The advent of multiple methods allows for a thorough approach to endothelial function. A comparison between these methods should take into account the pathophysiological relevance, validation and reproducibility, ability to predict events in diverse patient groups, ease of use, cost-effectiveness, and, ultimately, the ability to outperform traditional risk assessment tools.[109] The aforementioned techniques have a firm theoretical basis and address different facets of endothelial physiology. Older methods have been largely validated in multiple studies/subpopulations and proven useful in the reclassification of risk. Protocols have been largely standardized and this has resulted in reproducible measurements. However, a consensus has yet to be reached for most of the ex-vivo methods. All of the techniques are acceptable to the patient; learning curves and the necessity for special equipment may hamper the adoption of the most elaborate techniques in everyday clinical practice. Finally, the incremental predictive value has been established for all but the most recently developed ones. While no single method should be seen as an overall winner, it is nevertheless clear that they all add up to better prevention and, eventually, reversal of cardiovascular disease. Evaluation of endothelial function appears as an appealing adjunct for risk stratification. However, before its clinical implementation, it is essential that studies will address highly relevant questions, including which groups will benefit the most from such techniques and whether any clinical benefit from a specific therapeutic strategy is mediated through an improvement in endothelial function.

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## REFERENCES

1. Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, et al. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. N Engl J Med 1986; 315(17): 1046–1051.

2. Kim JW, Seo HS, Park JH, Na JO, Choi CU, Lim HE, et al. A prospective, randomized, 6-month comparison of the coronary vasomotor response associated with a zotarolimus- versus a sirolimus-eluting stent: differential recovery of coronary endothelial dysfunction. J Am Coll Cardiol 2009; 53(18): 1653–1659.

3. Husmann L, Gaemperli O, Schepis T, Scheffel H, Valenta I, Hoefflinghaus T, et al. Accuracy of quantitative coronary angiography with computed tomography and its dependency on plaque composition: plaque composition and accuracy of cardiac ct. Int J Cardiovasc Imaging 2008; 24(8): 895–904.

4. Terashima M, Nguyen PK, Rubin GD, Iribarren C, Courtney BK, Go AS, et al. Impaired coronary vasodilation by magnetic resonance angiography is associated with advanced coronary artery calcification. JACC Cardiovasc Imaging 2008; 1(2): 167–173.

5. Di Mario C, Heyndrickx GR, Prati F and Pijls NH. Chapter 8: Invasive imaging and haemodynamics. In: Camm AJ, Lu¨ scher TF, Serruys PW and European Society of Cardiology (eds) The ESC textbook of cardiovascular medicine, 2nd ed. Oxford, New York: Oxford University Press, 2009, p.xxiv, 1398 pp.

6. Prior JO, Schindler TH, Facta AD, Hernandez- Pampaloni M, Campisi R, Dahlbom M, et al. Determinants of myocardial blood flow response to cold pressor testing and pharmacologic vasodilation in healthy humans. Eur J Nucl Med Mol Imaging 2007; 34(1): 20–27.

7. Pijls NH, De Bruyne B, Smith L, Aarnoudse W, Barbato E, Bartunek J, et al. Coro-

nary thermodilution to assess flow reserve: validation in humans. Circulation 2002; 105(21): 2482–2486.

8. Aarnoudse W, Van't Veer M, Pijls NH, Ter Woorst J, Vercauteren S, Tonino P, et al. Direct volumetric blood flow measurement in coronary arteries by thermodilution. J Am Coll Cardiol 2007; 50(24): 2294–2304.

9. Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, et al. Prognostic value of coronary vascular endothelial dysfunction. Circulation 2002; 106(6): 653–658.

10. Targonski PV, Bonetti PO, Pumper GM, Higano ST, Holmes Jr DR and Lerman A. Coronary endothelial dysfunction is associated with an increased risk of cerebrovascular events. Circulation 2003; 107(22): 2805–2809.

11. Vassalli G, Gallino A, Kiowski W, Jiang Z, Turina M and Hess OM. Reduced coronary flow reserve during exercise in cardiac transplant recipients. Circulation 1997; 95(3): 607–613.

12. Joyner MJ, Dietz NM and Shepherd JT. From belfast to mayo and beyond: the use and future of plethysmography to study blood flow in human limbs. J Appl Physiol 2001; 91(6): 2431–2441. 13. Whitney RJ. The measurement of volume changes in human limbs. J Physiol 1953; 121(1): 1–27.

14. Hokanson DE, Sumner DS and Strandness Jr DE. An electrically calibrated plethysmograph for direct measurement of limb blood flow. IEEE Trans Biomed Eng 1975; 22(1): 25–29.

15. Panza JA, Quyyumi AA, Brush Jr JE and Epstein SE. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. N Engl J Med 1990; 323(1): 22–27.

16. Chowienczyk PJ, Watts GF, Cockcroft JR and Ritter JM. Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. Lancet 1992; 340(8833): 1430–1432.

17. Perticone F, Ceravolo R, Pujia A, Ventura G, Iacopino S, Scozzafava A, et al. Prognostic significance of endothelial dysfunction in hypertensive patients. Circulation 2001; 104(2): 191–196.

18. Heitzer T, Schlinzig T, Krohn K, Meinertz T and Munzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation 2001; 104(22): 2673–2678.

19. Fichtlscherer S, Breuer S and Zeiher AM. Prognostic value of systemic endothelial dysfunction in patients with acute coronary syndromes: further evidence for the existence of the ‘‘Vulnerable’’ patient. Circulation 2004; 110(14): 1926–1932.

20. Pohl U, Holtz J, Busse R and Bassenge E. Crucial role of endothelium in the vasodilator response to increased flow in vivo. Hypertension 1986; 8(1): 37–44.

21. Anderson EA and Mark AL. Flow-mediated and reflex changes in large peripheral artery tone in humans. Circulation 1989; 79(1): 93–100.

22. Mullen MJ, Kharbanda RK, Cross J, Donald AE, Taylor M, Vallance P, et al. Heterogenous nature of flowmediated dilatation in human conduit arteries in vivo: relevance to endothelial dysfunction in hypercholesterolemia. Circ Res 2001; 88(2): 145–151.

23. Leeson P, Thorne S, Donald A, Mullen M, Clarkson P and Deanfield J. Non-invasive measurement of endothelial function: effect on brachial artery dilatation of graded endothelial dependent and independent stimuli. Heart 1997; 78(1): 22–27.

24. Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager

MA, et al. Guidelines for the ultrasound assessment of endothelial-dependent flowmediated vasodilation of the brachial artery: a report of the international brachial artery reactivity task force. J Am Coll Cardiol 2002; 39(2): 257–265.

25. Sorensen KE, Celermajer DS, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Thomas O, et al. Noninvasive measurement of human endothelium dependent arterial responses: accuracy and reproducibility. Br Heart J 1995; 74(3): 247–253.

26. Donald AE, Halcox JP, Charakida M, Storry C, Wallace SM, Cole TJ, et al. Methodological approaches to optimize reproducibility and power in clinical studies of flowmediated dilation. J Am Coll Cardiol 2008; 51(20): 1959–1964.

27. Celermajer DS, Sorensen KE, Bull C, Robinson J and Deanfield JE. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. J Am Coll Cardiol 1994; 24(6): 1468–1474.

28. Halcox JP, Donald AE, Ellins E, Witte DR, Shipley MJ, Brunner EJ, et al. Endothelial function predicts progression of carotid intima-media thickness. Circulation 2009; 119(7): 1005–1012.

29. Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes Jr DR and Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation 2000; 101(9): 948–954.

30. Alexopoulos N, Vlachopoulos C, Aznaouridis K, Baou K, Vasiliadou C, Pietri P, et al. The acute effect of green tea consumption on endothelial function in healthy individuals. Eur J Cardiovasc Prev Rehabil 2008; 15(3): 300–305.

31. Woo KS, Chook P, Yu CW, Sung RY, Qiao M, Leung SS, et al. Effects of diet and exercise on obesity-related vascular dysfunction in children. Circulation 2004; 109(16): 1981–1986.

32. Wilkinson IB and McEniery CM. Arterial stiffness, endothelial function and novel pharmacological approaches. Clin Exp Pharmacol Physiol 2004; 31(11): 795–799.

33. Nichols WW, Denardo SJ, Wilkinson IB, McEniery CM, Cockcroft J and O'Rourke MF. Effects of arterial stiffness, pulse wave velocity, and wave reflections on the central aortic pressure waveform. J Clin Hypertens (Greenwich) 2008; 10(4): 295–303.

34. Kelly RP, Gibbs HH, O'Rourke MF, Daley JE, Mang K, Morgan JJ, et al. Nitroglycerin has more favourable effects on left ventricular afterload than apparent from measurement of pressure in a peripheral artery. Eur Heart J 1990; 11(2): 138–144.

35. Wilkinson IB, MacCallum H, Cockcroft JR and Webb DJ. Inhibition of basal nitric oxide synthesis increases aortic augmentation index and pulse wave velocity in vivo. Br J Clin Pharmacol 2002; 53(2): 189–192.

36. Klemsdal TO, Andersson TL, Matz J, Ferns GA, Gjesdal K and Anggard EE. Vitamin E restores endothelium dependent vasodilatation in cholesterol fed rabbits: in vivo measurements by photoplethysmography. Cardiovasc Res 1994; 28(9): 1397–1402.

37. Chowienczyk PJ, Kelly RP, MacCallum H, Millasseau SC, Andersson TL, Gosling RG, et al. Photoplethysmographic assessment of pulse wave reflection: blunted response to endothelium-dependent beta2- adrenergic vasodilation in type ii diabetes mellitus. J Am Coll Cardiol 1999; 34(7): 2007–2014.

38. Wilkinson IB, Hall IR, MacCallum H, Mackenzie IS, McEniery CM, van der Arend BJ, et al. Pulse-wave analysis: clinical evaluation of a noninvasive, widely applicable method for assessing endothelial function. Arterioscler Thromb Vasc Biol 2002; 22(1): 147–152.

39. Hayward CS, Kraidly M, Webb CM and Collins P. Assessment of endothelial function using peripheral waveform analysis: a clinical application. J Am Coll Cardiol 2002;

40(3): 521–528.

40. Kals J, Kampus P, Kals M, Pulges A, Teesalu R and Zilmer M. Effects of stimulation of nitric oxide synthesis on large artery stiffness in patients with peripheral arterial disease. Atherosclerosis 2006; 185(2): 368–374.

41. Wallberg-Jonsson S, Caidahl K, Klintland N, Nyberg G and Rantapaa-Dahlqvist S. Increased arterial stiffness and indication of endothelial dysfunction in long-standing rheumatoid arthritis. Scand J Rheumatol 2008; 37(1): 1–5.

42. Lind L, Fors N, Hall J, Marttala K and Stenborg A. A comparison of three different methods to evaluate endothelium-dependent vasodilation in the elderly: the prospective investigation of the vasculature in uppsala seniors (pivus) study. Arterioscler Thromb Vasc Biol 2005; 25(11): 2368–2375.

43. Kuvin JT, Patel AR, Sliney KA, Pandian NG, Sheffy J, Schnall RP, et al. Assessment of peripheral vascular endothelial function with finger arterial pulse wave amplitude. Am Heart J 2003; 146(1): 168–174.

44. Noon JP, Haynes WG, Webb DJ and Shore AC. Local inhibition of nitric oxide generation in man reduces blood flow in finger pulp but not in hand dorsum skin. J Physiol 1996; 490(Pt 2): 501–508.

45. Nohria A, Gerhard-Herman M, Creager MA, Hurley S, Mitra D and Ganz P. Role of nitric oxide in the regulation of digital pulse volume amplitude in humans. J Appl Physiol 2006; 101(2): 545–548.

46. Bonetti PO, Pumper GM, Higano ST, Holmes Jr DR, Kuvin JT and Lerman A. Noninvasive identification of patients with early coronary atherosclerosis byassessment of digital reactive hyperemia. J Am Coll Cardiol 2004; 44(11): 2137–2141.

47. de Jongh RT, Serne EH, IJzerman RG, de Vries G and Stehouwer CD. Impaired mi-

crovascular function in obesity: implications for obesity-associated microangiopathy, hypertension, and insulin resistance. Circulation 2004; 109(21): 2529–2535.

48. Mitchell GF, Vita JA, Larson MG, Parise H, Keyes MJ, Warner E, et al. Cross-sectional relations of peripheral microvascular function, cardiovascular disease risk factors, and aortic stiffness: the framingham heart study. Circulation 2005; 112(24): 3722–3728.

49. Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK, et al. (–)-epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. Proc Natl Acad Sci U S A 2006; 103(4): 1024–1029.

50. Rubinshtein R, Kuvin JT, Soffler M, Lennon RJ, Lavi S, Nelson RE, et al. Assessment of endothelial function by non-invasive peripheral arterial tonometry predicts late cardiovascular adverse events. Eur Heart J 2010; 31(9): 1142–1148.

51. Gush RJ and King TA. Discrimination of capillary and arterio-venular blood flow in skin by laser doppler flowmetry. Med Biol Eng Comput 1991; 29(4): 387–392.

52. Khan F, Patterson D, Belch JJ, Hirata K and Lang CC. Relationship between peripheral and coronary function using laser doppler imaging and transthoracic echocardiography. Clin Sci (Lond) 2008; 115(9): 295–300.

53. Fredriksson I, Larsson M and Stromberg T. Measurement depth and volume in laser doppler flowmetry. Microvasc Res 2009; 78(1): 4–13.

54. Ozbebit FY, Esen F, Gulec S and Esen H. Evaluation of forearm microvascular blood flow regulation by laser doppler flowmetry, iontophoresis, and curve analysis: contribution of axon reflex. Microvasc Res 2004; 67(3): 207–214.

55. Durand S, Tartas M, Bouye P, Koitka A, Saumet JL and Abraham P. Prostaglandins participate in the late phase of the vascular response to acetylcholine iontophoresis in

humans. J Physiol 2004; 561(Pt 3): 811–819.

56. Norman M and Martin H. Preterm birth attenuates association between low birth weight and endothelial dysfunction. Circulation 2003; 108(8): 996–1001.

57. Trzepizur W, Gagnadoux F, Abraham P, Rousseau P, Meslier N, Saumet JL, et al. Microvascular endothelial function in obstructive sleep apnea: impact of continuous positive airway pressure and mandibular advancement. Sleep Med 2009; 10(7): 746–752.

58. Caballero AE, Arora S, Saouaf R, Lim SC, Smakowski P, Park JY, et al. Microvascular and macrovascular reactivity is reduced in subjects at risk for type 2 diabetes. Diabetes 1999; 48(9): 1856–1862.

59. Khan F, Elhadd TA, Greene SA and Belch JJ. Impaired skin microvascular function in children, adolescents, and young adults with type 1 diabetes. Diabetes Care 2000; 23(2): 215–220.

60. Andreassen AK, Gullestad L, Holm T, Simonsen S and Kvernebo K. Endothelium-dependent vasodilation of the skin microcirculation in heart transplant recipients. Clin Transplant 1998; 12(4): 324–332.

61. Abularrage CJ, Sidawy AN, Aidinian G, Singh N, Weiswasser JM and Arora S. Evaluation of the microcirculation in vascular disease. J Vasc Surg 2005; 42(3): 574–581.

62. Kubli S, Feihl F and Waeber B. Beta-blockade with nebivolol enhances the acetylcholine-induced cutaneous vasodilation. Clin Pharmacol Ther 2001; 69(4): 238–244.

63. Rossi M, Carpi A, Di Maria C, Franzoni F, Galetta F and Santoro G. Skin blood flowmotion and microvascular reactivity investigation in hypercholesterolemic patients without clinically manifest arterial diseases. Physiol Res 2009; 58(1): 39–47.

64. Settergren M, Pernow J, Brismar K, Jorneskog G and Kalani M. Endothelin-a receptor blockade increases nutritive skin capillary circulation in patients with type 2 diabetes and microangiopathy. J Vasc Res 2008; 45(4): 295–302.

65. Vallance P, Leone A, Calver A, Collier J and Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet 1992; 339(8793): 572–575.

66. Iribarren C, Husson G, Sydow K, Wang BY, Sidney S and Cooke JP. Asymmetric dimethyl-arginine and coronary artery calcification in young adults entering middle age: the cardia study. Eur J Cardiovasc Prev Rehabil 2007; 14(2): 222–229.

67. Juonala M, Viikari JS, Alfthan G, Marniemi J, Kahonen M, Taittonen L, et al. Brachial artery flow-mediated dilation and asymmetrical dimethylarginine in the cardiovascular risk in young finns study. Circulation 2007; 116(12): 1367–1373.

68. Schnabel R, Blankenberg S, Lubos E, Lackner KJ, Rupprecht HJ, Espinola-Klein C, et al. Asymmetric dimethylarginine and the risk of cardiovascular events and death in patients with coronary artery disease: results from the atherogene study. Circ Res 2005; 97(5): e53–59.

69. Leong T, Zylberstein D, Graham I, Lissner L, Ward D, Fogarty J, et al. Asymmetric dimethylarginine independently predicts fatal and nonfatal myocardial infarction and stroke in women: 24-year follow-up of the population study of women in gothenburg. Arterioscler Thromb Vasc Biol 2008; 28(5): 961–967.

70. Boger RH, Sullivan LM, Schwedhelm E, Wang TJ, Maas R, Benjamin EJ, et al. Plasma asymmetric dimethylarginine and incidence of cardiovascular disease and death in the community. Circulation 2009; 119(12): 1592–1600.

71. Tsimikas S. In vivo markers of oxidative stress and therapeutic interventions. Am J

Cardiol 2008; 101(10A): 34D–42D.

72. Meisinger C, Baumert J, Khuseyinova N, Loewel H and Koenig W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. Circulation 2005; 112(5): 651–657.

73. Wu T, Willett WC, Rifai N, Shai I, Manson JE and Rimm EB. Is plasma oxidized low-density lipoprotein, measured with the widely used antibody 4e6, anindependent predictor of coronary heart disease among US. men and women? J Am Coll Cardiol 2006; 48(5): 973–979.

74. Sorrentino SA, Besler C, Rohrer L, Meyer M, Heinrich K, Bahlmann FH, et al. Endothelial-vasoprotective effects of high-density lipoprotein are impaired in patients with type 2 diabetes mellitus but are improved after extended-release niacin therapy. Circulation 2010; 121(1): 110–122.

75. Whincup PH, Danesh J, Walker M, Lennon L, Thomson A, Appleby P, et al. Von Willebrand factor and coronary heart disease: prospective study and meta-analysis. Eur Heart J 2002; 23(22): 1764–1770.

76. Hugel B, Martinez MC, Kunzelmann C and Freyssinet JM. Membrane microparticles: two sides of the coin. Physiology (Bethesda) 2005; 20: 22–27.

77. Chironi GN, Boulanger CM, Simon A, Dignat-George F, Freyssinet JM and Tedgui A. Endothelial microparticles in diseases. Cell Tissue Res 2009; 335(1): 143–151.

78. Wang JM, Wang Y, Huang JY, Yang Z, Chen L, Wang LC, et al. C-reactive protein-induced endothelial microparticle generation in huvecs is related to bh4-dependent no formation. J Vasc Res 2007; 44(3): 241–248.

79. Boulanger CM, Amabile N, Guerin AP, Pannier B, Leroyer AS, Mallat CN, et al. In

vivo shear stress determines circulating levels of endothelial microparticles in end-stage renal disease. Hypertension 2007; 49(4): 902–908.

80. Boulanger CM, Amabile N and Tedgui A. Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease. Hypertension 2006; 48(2): 180–186.

81. Amabile N, Guerin AP, Leroyer A, Mallat Z, Nguyen C, Boddaert J, et al. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. J Am Soc Nephrol 2005; 16(11): 3381–3388.

82. Esposito K, Ciotola M, Giugliano F, Schisano B, Improta L, Improta MR, et al. Endothelial microparticles correlate with erectile dysfunction in diabetic men. Int J Impot Res 2007; 19(2): 161–166.

83. Esposito K, Ciotola M, Schisano B, Gualdiero R, Sardelli L, Misso L, et al. Endothelial microparticles correlate with endothelial dysfunction in obese women. J Clin Endocrinol Metab 2006; 91(9): 3676–3679.

84. Mallat Z, Benamer H, Hugel B, Benessiano J, Steg PG, Freyssinet JM, et al. Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation 2000; 101(8): 841–843.

85. Koga H, Sugiyama S, Kugiyama K, Watanabe K, Fukushima H, Tanaka T, et al. Elevated levels of vecadherin- positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. J Am Coll Cardiol 2005; 45(10): 1622–1630.

86. Bernal-Mizrachi L, Jy W, Jimenez JJ, Pastor J, Mauro LM, Horstman LL, et al. High levels of circulating endothelial microparticles in patients with acute coronary

syndromes. Am Heart J 2003; 145(6): 962–970.

87. Amabile N, Heiss C, Real WM, Minasi P, McGlothlin D, Rame EJ, et al. Circulating endothelial microparticle levels predict hemodynamic severity of pulmonary hypertension. Am J Respir Crit Care Med 2008; 177(11): 1268–1275.

88. Boulanger CM, Scoazec A, Ebrahimian T, Henry P, Mathieu E, Tedgui A, et al. Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction. Circulation 2001; 104(22): 2649–2652.

89. Schwartz SM and Benditt EP. Clustering of replicating cells in aortic endothelium. Proc Natl Acad Sci U S A 1976; 73(2): 651–653.

90. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999; 85(3): 221–228.

91. Duda DG, Cohen KS, Scadden DT and Jain RK. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. Nat Protoc 2007; 2(4): 805–810.

92. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 2007; 109(5): 1801–1809.

93. Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, et al. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. Circulation 2005; 111(22): 2981–2987.

94. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, et al. Hmg-coa reductase inhibitors (statins) increase endothelial progenitor cells via the pi 3-

kinase/akt pathway. J Clin Invest 2001; 108(3): 391–397.

95. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003; 348(7): 593–600. 96. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 2005; 353(10): 999–1007.

97. Jung C, Rafnsson A, Shemyakin A, Bohm F and Pernow J. Different subpopulations of endothelial progenitor cells and circulating apoptotic progenitor cells in patients with vascular disease and diabetes. Int J Cardiol 2010; 143(3): 368–372.

98. Desai A, Glaser A, Liu D, Raghavachari N, Blum A, Zalos G, et al. Microarray-based characterization of a colony assay used to investigate endothelial progenitor cells and relevance to endothelial function in humans. Arterioscler Thromb Vasc Biol 2009; 29(1): 121–127.

99. Erbs S, Linke A, Schachinger V, Assmus B, Thiele H, Diederich KW, et al. Restoration of microvascular function in the infarct-related artery by intracoronary transplantation of bone marrow progenitor cells in patients with acute myocardial infarction: the doppler substudy of the reinfusion of enriched progenitor cells and infarctremodeling in acute myocardial infarction (repair-ami) trial. Circulation 2007; 116(4): 366–374.

100. Ikonomidis I, Papadimitriou C, Vamvakou G, Katsichti P, Venetsanou K, Stamatelopoulos K, et al. Treatment with granulocyte colony stimulating factor is associated with improvement in endothelial function. Growth Factors 2008; 26(3): 117–124.

101. van den Berg BM, Nieuwdorp M, Stroes ES and Vink H: Glycocalyx and endothelial (dys) function: from mice to men. Pharmacol Rep 2006; 58 (Suppl): 75–80.

102. Van Teeffelen JW, Brands J, Jansen C, Spaan JA and Vink H. Heparin impairs

glycocalyx barrier properties and attenuates shear dependent vasodilation in mice. Hypertension 2007; 50(1): 261–267.

103. Bruegger D, Rehm M, Jacob M, Chappell D, Stoeckelhuber M, Welsch U, et al. Exogenous nitric oxide requires an endothelial glycocalyx to prevent postischemic coronary vascular leak in guinea pig hearts. Crit Care 2008; 12(3): R73.

104. Proudfoot AE, Handel TM, Johnson Z, Lau EK, LiWang P, Clark-Lewis I, et al. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. Proc Natl Acad Sci U S A 2003; 100(4): 1885–1890.

105. Nieuwdorp M, van Haeften TW, Gouverneur MC, Mooij HL, van Lieshout MH, Levi M, et al. Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo. Diabetes 2006; 55(2): 480–486.

106. Nieuwdorp M, Meuwese MC, Mooij HL, van Lieshout MH, Hayden A, Levi M, et al. Tumor necrosis factoralpha inhibition protects against endotoxin-induced endothelial glycocalyx perturbation. Atherosclerosis 2009; 202(1): 296–303.

107. Nieuwdorp M, Holleman F, de Groot E, Vink H, Gort J, Kontush A, et al. Perturbation of hyaluronan metabolism predisposes patients with type 1 diabetes mellitus to atherosclerosis. Diabetologia 2007; 50(6): 1288–1293.

108. Meuwese MC, Mooij HL, Nieuwdorp M, van Lith B, Marck R, Vink H, et al. Partial recovery of the endothelial glycocalyx upon rosuvastatin therapy in patients with heterozygous familial hypercholesterolemia. J Lipid Res 2009; 50(1): 148–153.

109. Romanens M, Ackermann F, Spence JD, Darioli R, RodondiN, Corti R, et al. Improvement of cardiovascular risk prediction: Time to review current knowledge, debates, and fundamentals on how to assess test characteristics. Eur J Cardiovasc Prev

Rehabil 2010; 17(1): 18–23.

## FIGURES

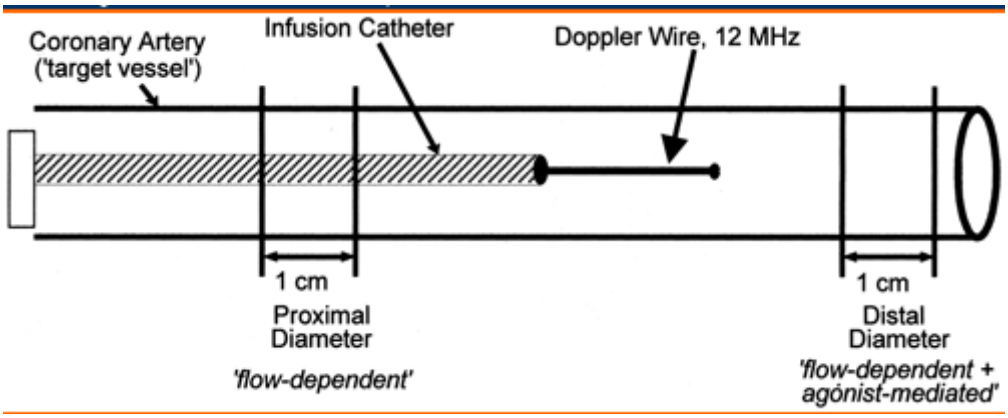
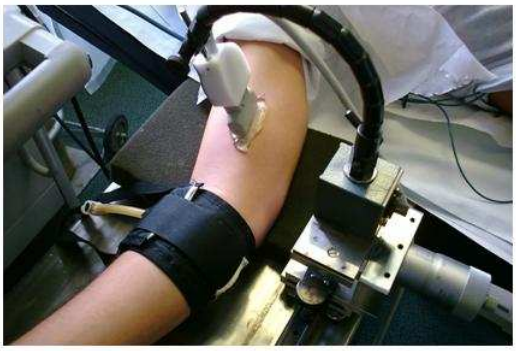



**Figure 1:** Schematic of a Doppler guidewire including an infusion catheter and a 12 MHz Doppler wire. The coaxial position of the wire in the vessel lumen can be appreciated. Also shown are two 1 cm-vessel segments where changes in vessel diameter are measured proximal and distal to the injection site of the vasodilator agent. Source: E-xerc Sport Sci Rev © American College of Sports Medicine



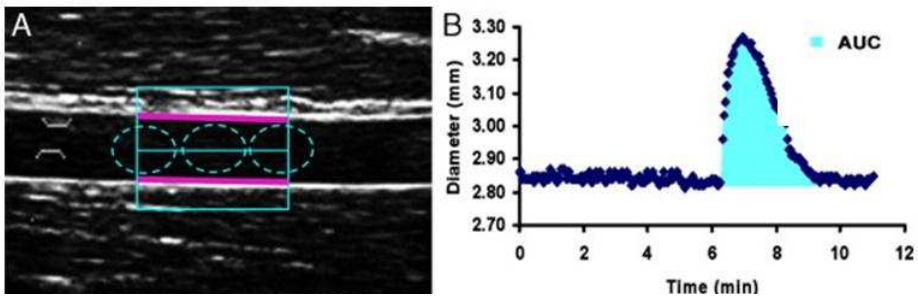


**Figure 2:** Flow mediated dilatatation. The top figure illustrates the optimal set-up for FMD measurements. The bottom figures demonstrate brachial artery in a longitudinal section (A) and the output using the automated analysis software (Brachial Analyser) (B).

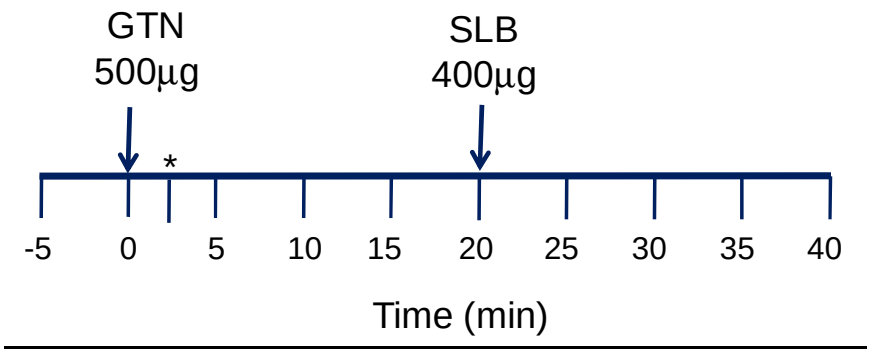



**Figure 3:** Schema for PWA assessment of endothelial function. SLB = salbutamol

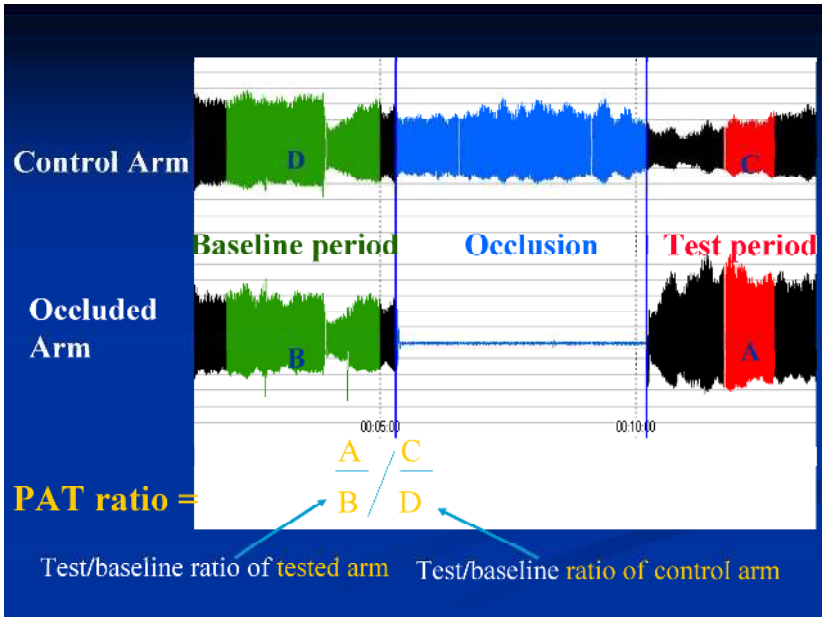



**Figure 4:** Typical recordings from the EndoPat. Estimation of PAT ratio after correction of the reactive hyperemia index for the control arm. Probe 1 corresponds to the oc-

cluded arm and probe 2 to the control arm. The blue colored interval corresponds to the duration of occlusion in the test arm**.**

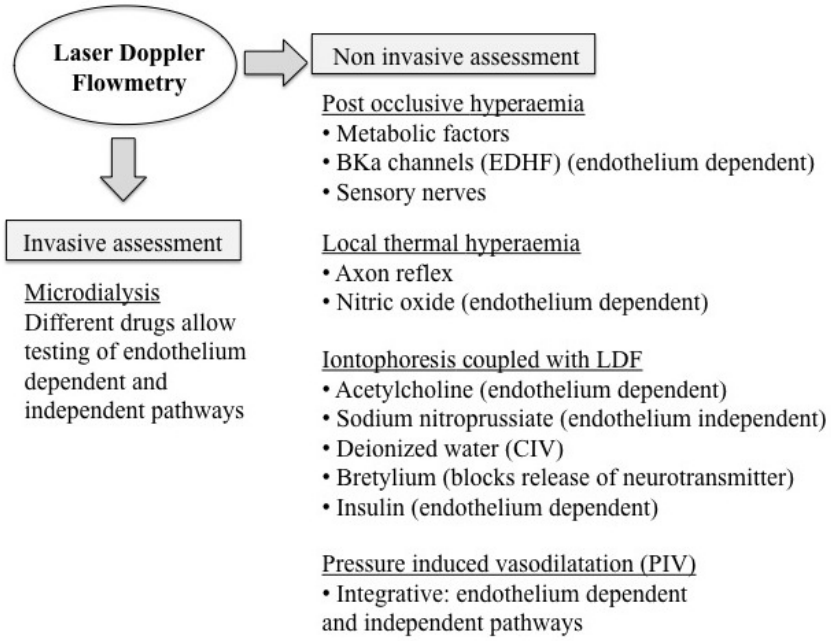



**Figure 5:** Types of investigation with Laser Doppler flowmetry.

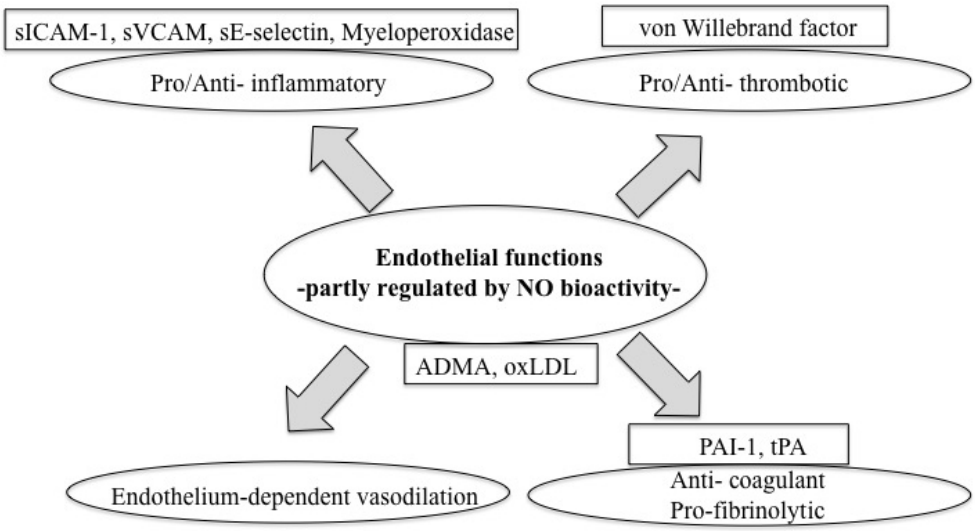



**Figure 6:** Biomarkers for endothelial functions

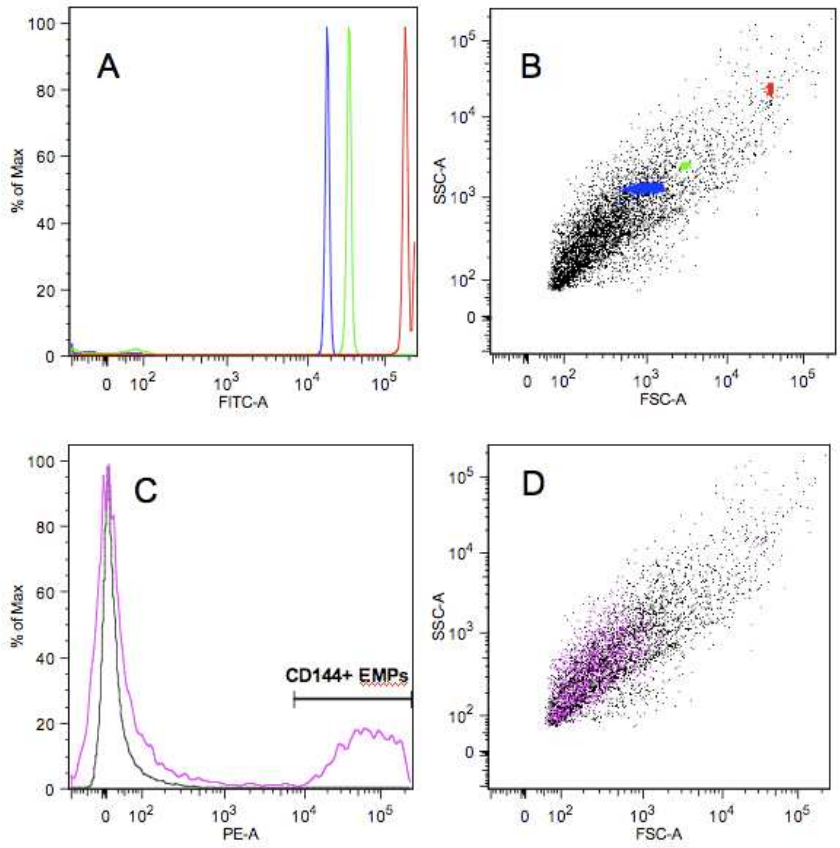



**Figure 7:** Representative flow cytometry experiment aiming at identifying circulating endothelial CD144+ MPs in acute myocardial infarction. **A**: identification of fluorescent synthetic beads within the MP size range (Blue= 0.5 µm; Green= 0.8 µm and Red= 3 µm in diameter). **B**: forward scatter (FS) vs. side scatter (SS) dot plot representation showing MPs (black) and synthetic beads. **C**: Representative flow cytometry fluorescence histogram showing the presence of CD144 (VE-cadherin) on circulating MPs (purple line). The black line corresponds to negative control (isotypic IgGs). Platelet-free plasma was obtained as indicated above and exposed to anti-VE-cadherin monoclonal antibody coupled to phycoerythrin (PE; purple line) or its isotypic control (black line). **D**: forward scatter (FS) vs. side scatter (SS) dot plot representation showing endothelial CD144+MPs (purple dots) in the platelet-free plasma of a patients with acute myocardial infarction.

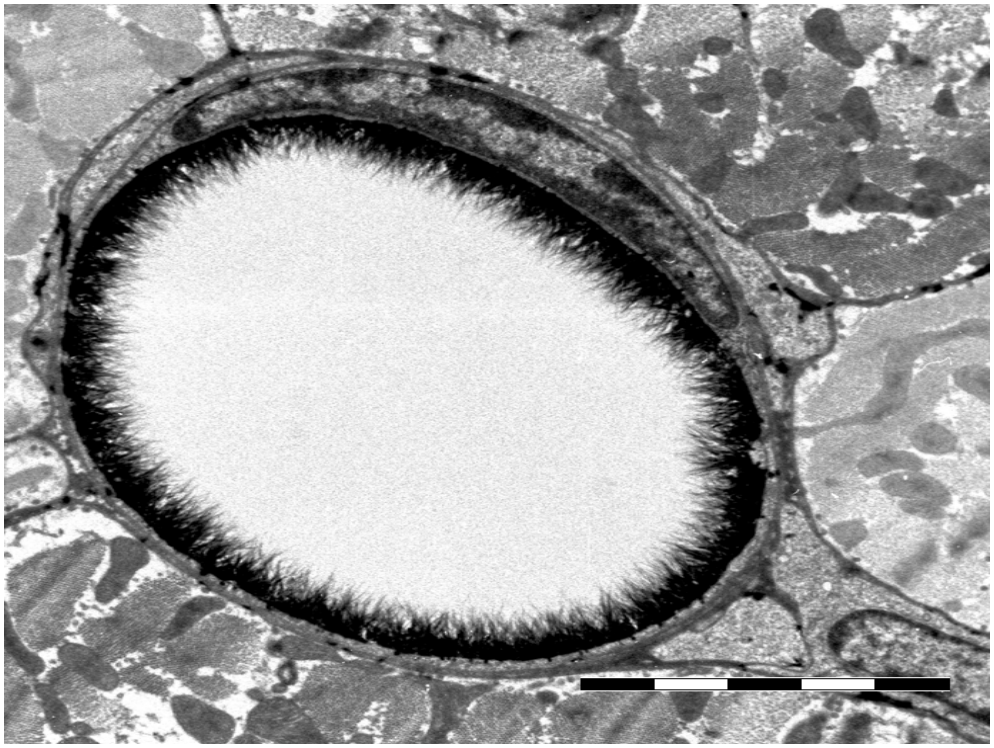


**Figure 8:** Electron microscopy image of coronary endothelial glycocalyx (courtesy of B. van den Berg, Maastricht University).

## TABLES

| Molecule | Function | Synonym | Distribution |
|---|---|---|---|
| CD14 | Receptor/signaling | LPS receptor | Monocytes, macrophages and neutrophils |
| CD31 | Adhesion | PECAM-1 | Very widely distributed |
| CD34 | Adhesion (precise function unclear) | | Leukocytes at various stages of differentiation, especially haemopoietic or endothelial progenitors. Also on numerous neoplastic cells. |
| CD45 | Signaling | Leukocyte common antigen | 'Mature' and neoplastic leukocytes |
| CD69E | Adhesion | E-selectin | Endothelial cells |
| CD105 | Signaling | TFG-β receptor/endoglin | Endothelial cells, activated monocytes, pre-B lymphocytes |
| CD133 | Unclear | Promin-1 | Haematopoietic stem cells, endothelial progenitor cells, some neuronal cells |
| CD144 | Adhesion and sig- | VE-Cadherin | Endothelial cells |

| | | | |
|---|---|---|---|
| | naling | | |
| CD146 | Adhesion | Mel-CAM/MUC-18 | Mature endothelial cells, T lymphocytes, some neoplastic cells. |
| CD202b | Signaling | Tie-2 (angiopoietin receptor) | Endothelial cells |
| CD309 | Signaling | VEGFR2/KDR/Flk-1 | Endothelial cells |

**Table 1** Cell surface molecules used to define endotheliod cells in vivo and in vitro.

| Name of cell | Abbreviation |
|---|---|
| Angioblast | - |
| Blood outgrowth endothelial cell[#] | BOEC |
| Circulating angioblast | - |
| Circulating angiogenic cell | CAC* |
| Circulating endothelial cell | CEC |
| Circulating endothelial progenitor cell | CEPC |
| Endothelial cell colony forming unit | CFU-EC*[#] |
| Culture modified mononuclear cells | CMMC* |
| Circulating progenitor cell | CPC |
| Endothelial cell | EC*[#] |
| Endothelial-like cell | ELC |
| Endothelial colony forming cell | ECFC |
| Endothelial progenitor cell | EPC*[#] |
| Endothelial outgrowth cell | EOC |
| Haemopoietic precursor cell | HPC |
| Haemopoietic stem cell | HSC |
| Multipotent adult progenitor cell | MAPC |

**Table 2** Endotheliod cells. All of these cells are defined by different but often overlapping markers in vivo and in vitro. *EC-like cells, #EOCs