Chapter 17

The Markers of Endothelial Activation

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Additional information is available at the end of the chapter

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Abstract

Biomarkers are biological indicators of processes that are part of ethiopathogenesis of the diseases, and can, but do not have to be causal to diseases. One very important question is how specific and sensitive the marker is, since one molecule can appear in many conditions. Biomarkers of endothelial cell activation can be very diverse, from biochemical/metabolic to functional biomarkers. Activation of endothelial cells is part of physiological as well as pathophysiological response of cardiovascular system in conditions as physical activity, growth, pregnancy and in all cardiometabolic diseases (e.g., hypertension, diabetes mellitus, autoimmune inflammatory diseases, coronary artery disease, atherosclerosis, ischemia and reperfusion, etc.). During activation, there is a change in endothelial cell morphology and function, which could be a defensive response of endothelium to provoking factor or could lead to increased risk for the injury and end organ damage. This chapter aims to overview current knowledge on established biomarkers of normal and disease-related endothelial activation and to provide information on novel, potential biomarkers in common cardiometabolic diseases.

Keywords: endothelial activation, biomarkers, laser Doppler flowmetry, flow mediated dilation, pregnancy, exercise, cardiometabolic diseases, functional markers, nitric oxide, prostaglandins

1. Introduction

Vascular endothelium has a critical role in maintaining vascular tone, and changes in vascular flow are in complex interactions with endothelium. The importance of this particular function of the endothelium manifests in the fact that the term "endothelial function" is usually used to

describe the ability of endothelium to release vasoactive substances and thereby regulate blood flow. The basic principle of vascular function is that healthy blood vessels dilate normally and the diseased blood vessels exhibit a dysfunctional vasodilation. Therefore, all methods for endothelial function assessment are based on the ability of endothelium to respond (vasodilation or vasoconstriction) to a specific stimulus (vascular occlusion, pharmacological vasodilators, heating, etc.).

Biomarkers are biological indicators of processes that are part of etiology of the diseases, and can, but do not have to be causal to diseases. One very important question is how specific and sensitive the marker is, since one molecule can appear in many conditions. Biomarkers of endothelial cell activation can be very diverse: biochemical/metabolic (such as plasma glucose, lipids, cytokines, asymmetric dimethylarginine (ADMA), high sensitive C-reactive protein (hsCRP), myeloperoxidase (MPO), cell adhesion molecules (CAMs), markers of coagulability, markers of oxidative stress, chemokines, microparticles, endothelial progenitor cells), functional biomarkers (such as flow-mediated dilation and other types of flowmetry, arteriographic measurements of vascular function) and structure (e.g., CIMT—carotid intima-media thickness, angiogenesis, or rarefaction).

2. Biochemical biomarkers of vascular (endothelial) function

Over the last three decades, a number of methodological approaches were developed in order to evaluate and measure (patho)physiological function of the endothelium in humans [1, 2]. Evidently, these new methods intensified research and brought novelties in the field of vascular physiology and pathophysiology, but still are not implemented as clinical tools in daily practice. The approaches for endothelial function assessment were designed to provide insight into vascular/endothelial function in different sites (vascular beds) and different blood vessel types (conductive, resistant, and microcirculation). Earlier methods were more invasive (e.g., intracoronary infusion of acetylcholine (ACh), and later developed techniques that were less invasive have focused on peripheral circulation (forearm circulation) as a surrogate for coronary arteries [3–5]. As expected, all of these methods have their advantages and accepted limitations, and neither of the developed methods does present the absolute standard for the evaluation of endothelial function, in both macro- and microcirculation.

There is an extensive body of evidence reporting that generalized endothelial dysfunction exhibited virtually in every arterial bed presents an early manifestation of a variety of cardiovascular diseases (CVDs) [6, 7]. Still, when investigating endothelial function in different CVDs, diverse (patho)physiological role of large conductance vessels and small microvasculature should be considered.

There are many various molecules which have been denoted as vascular or endothelial markers, e.g., lipids, cytokines, ADMA, hsCRP, MPO, CAMs, markers of coagulability, markers of oxidative stress, chemokines, microparticles, and endothelial progenitor cells. It has been demonstrated that reduced bioavailability of nitric oxide (NO) plays a central role in impaired vascular/endothelial response (endothelial dysfunction) in conduit arteries, while NO in the microcirculation primarily modulates tissue metabolism [8]. On the other hand, a number of

studies indicate that endothelium-derived hyperpolarizing factor (EDHF) plays a major role in vasodilation in skin microcirculation [9], whereas the results are still conflicting concerning the implication of prostaglandins [10–12]. A study on coronary endothelial function in young smokers reported that they had epicardial coronary endothelial dysfunction but preserved microvascular endothelial function [13].

2.1. Biomarkers in pregnancy

The importance of maternal vascular adaptation to pregnancy is to increase blood flow and to assure the proper development of the fetus. Several possible biochemical biomarkers have been proposed to evaluate vascular/endothelial function in pregnancy. First among them is NO, one of most important endothelial vasodilators, which is produced by NO synthase (NOS). It is well accepted that NOS-3 expression levels are increased in uterine artery endothelium in pregnancy [14]. Prostacyclin (PGI2) also plays an important role in vasodilator response, and its concentration is elevated in pregnancy [15]. In order to estimate the real impact of prostacyclin on vascular tone, determination of thromboxane a2 (TXA2)/PGI2 ratio is needed. Since both TXA2 and PGI2 have very short half-life, only indirect measures can be made of stable metabolites in the blood (thromboxane b2 (TXB2) and 6-keto-prostaglandin F2a (6-ketoPGF2A)), and there is no technique which allows their monitoring in real time. It has been demonstrated that cyclooxygenase 1 (COX1) is upregulated in endothelial cells during pregnancy, and therefore induces a PGI2 increment [14]. EDHF is the third major player in endothelial vasodilation in pregnancy, causing smooth muscle relaxation. As it is not a single factor and there is still ongoing research to identify its specific components, it is described as spectrum of responses that are neither NO nor PGI2 mediated. Another limiting problem is that there is no appropriate method for its tracking. Although EDHF may seem as unnecessary pathway beside NO and PGI2, a number of studies showed an important role of EDHF in endothelium vasodilation in pregnancy, suggesting that without EDHF, there would not be sufficient blood flow to the fetus [16].

Endogenous eNOS inhibitor ADMA concentrations were found to be significantly lower in pregnant women. However, this did not explain the improved flow-mediated dilation (FMD) in the correlation analysis [17, 18]. Also, endothelial function in normal pregnancy was not attenuated despite the significant increase in hsCRP, and pregnancy-related changes in the concentrations of proinflammatory cytokines, e.g., tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), were nonsignificant [19].

2.2. Biomarkers in exercise

It has been reported that the regulation of the NO-dependent pathway presents a key mechanism mediating endothelial adaptations to shear stress, including increased NO synthesis, increased expression and activity of antioxidative enzymes (e.g., superoxide dismutase (SOD) and catalase), and decreased oxidative stress level (reactive oxygen species (ROS) production) which all increases NO bioavailability. However, recent studies demonstrated that COX-dependent pathway and increased PGI2 synthesis take part in endothelial adaptations to shear stress, as well. Furthermore, a growing body of evidence suggest that increased shear stress generated by increased blood flow during exercise, presents a prime signal for

decreased level of vasoconstrictor endothelin 1 (ET1), and inflammatory markers such as vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemotactic protein 1 (MCP-1) level [20]. Furthermore, increased endothelial NOS (eNOS) gene expression has been proposed to be a marker of anterograde shear stress-induced endothelial activation (result of repeated episodic increase in blood flow during exercise), and to have anti-atherogenic effect in endothelial cell cultures [21, 22].

On the other hand, rhythmic stretching (cyclic strain) provoked by systolic blood pressure changes during exercise affects endothelial cell growth and NO- and EDHF-dependent vasodilation pathway, and its effect depends on the blood pressure increment during exercise (e.g., >135 mmHg elicits inhibition of endothelial cell growth) [23]. Surprisingly, further studies on endothelial cell cultures have reported that rhythmic stretching can induce ROS production and increase the expression of cell adhesion molecules. On the other hand, ROS produced by cyclic strain may indirectly increase expression of eNOS [24]. It became evident that the time of exposure to high blood pressure/cyclic strain (continuous or pulsatile) is crucial for its final effect on endothelial function. Brief increases in blood pressure and ROS production associated with bouts of exercise may signal an increase in eNOS production and other beneficial effects resulting in improved endothelial function. Chronic increases in cyclic strain (e.g., hypertension) may elevate ROS chronically and finally provoke development of endothelial dysfunction. Thus, beside abovementioned endothelial biomarkers of inflammation and endothelial dysfunction, measurement of oxidative stress level and antioxidant capacity present suitable and commonly used markers of endothelial response to different exercise modes and patterns (shear stress) in both health and disease.

2.3. Biomarkers in cardiometabolic diseases

Oxidation of low density lipoproteins (oxLDL) and NO synthesis contribute to endothelial dysfunction, vascular aging, and disease. OxLDL and NO exert contradictory actions within the vascular endothelium such as: leukocyte adhesion, platelet aggregation, and vascular smooth muscle cell proliferation and migration [25, 26]. While oxLDL—an oxidative stress biomarker—has been identified as a pro-atherogenic risk factor for coronary artery disease (CAD), NO is a free radical signal-transducing molecule that maintains vasodilation, modulates *in vitro* lipid peroxidation reactions and alters pro-inflammatrory gene expression. Both are part of complex atherosclerotic process, from initiation to plaque destabilization and coronary artery disease [25, 26].

As already mentioned, ADMA is an endogenous inhibitor of NO synthase [27] and thus may cause endothelial dysfunction [28]. Increased plasma levels of ADMA are related with hyperlipidemia, hypertension, coronary artery disease, unstable angina, stroke and end-stage renal disease and diabetes [28]. Reduced plasma levels of ADMA after percutaneous coronary intervention could be indicative of a reduced risk of recurrent cardiovascular events. Although ADMA was significantly associated with all-cause mortality in patients with acute coronary syndrome and ischemic heart disease, there is no clear association between ADMA and cardiovascular disease incidence [29]. Type II diabetes has been associated with increased ADMA levels. ADMA and NO have been found to be significant determinants of insulin resistance [30]. A study performed in type 2 diabetes patients that used antidiabetic metformin for

3 months showed reduced serum ADMA levels for 30% [31]. Another study, from Stuhlinger et al. found that rosiglitazone reduced the level of ADMA by 30% in seven insulin-resistant non-diabetic hypertensive individuals [32].

Toll-like receptors (TLRs), such as toll-like receptors TLR2 and TLR4 have been found to have elevated expression in T2DM patients, which could be a possible underlying mechanism of inflammation in T2DM [33]. TLR-2 and TLR-4 activation has also been found in murine models of atherosclerosis [33, 34]. There are many unanswered questions: the consequences of activation/blockade of TLRs in atherosclerosis, relationship between innate and adaptive responses in atherosclerosis, and mechanistic insight on the intricate balance of direct and risk factor-mediated effects of TLRs in CVD [33, 34].

The over expression of TNF-alpha and its inflammatory and immunomodulatory effects have been implicated in the pathogenesis of CAD and myocardial dysfunction. Cardiovascular complications may be influenced by TNF-alpha gene polymorphisms. Certain studies failed to find a significant association between the TNF-alpha gene polymorphisms and CVD [35]. Further studies are required to resolve this controversy.

IL-6 is associated with the process of inflammation and coronary artery disease. Patients with high levels of IL-6 show worse in-hospital outcome following treatment in case of unstable angina. An association has been shown between the IL-6 promoter polymorphism −174G/C and hypertension, left ventricular hypertrophy and ischemic heart disease CAD [35].

Endothelial cells also express chemotactic factors: MCP-1, proinflammatory cytokines (macrophage colony-stimulating factor) and tumor necrosis factor-beta (TNF-β) [36]. Hyperglycemia promotes MCP-1 expression in vascular endothelial cells and has a pivotal role in the pathogenesis of diabetic vasculopathy [37]. Patients with diabetes mellitus or obesity have increased circulating levels of inflammatory markers, including C reactive protein (CRP), TNF-α, and IL-6 [38–40]. Blood level of CRP, as independent predictor of diabetes, is increased in both Type I and Type II diabetes [41, 42]. TNF-α can induce cytokines such as IL-6 which regulates the expression of CRP. They can impair endothelial function and contribute to atherothrombosis especially in patients with Type II diabetes, alone or in combination [43]. It was also found in male diabetic patients that increased levels of inflammatory markers predict cardiovascular risk in diabetic patients [44].

Microparticles, the membrane vesicles released by various cell types and circulating endothelial cells represent novel biomarkers of endothelial injury, associated with atherosclerosis and related complications (thrombosis, inflammation, and apoptosis). Microparticles are suggested to be biomarkers of vascular injury and inflammation [45]. Changes in circulating levels of microparticles might give an important clinical information in healthy subjects or patients with CVDs as a surrogate marker of vascular function, but it is still not clear whether it is a cause or effect of atherosclerosis [45].

Endocan or endothelial cell specific molecule-1 (ESM-1) is a novel endothelium-derived soluble proteoglycan [46]. It binds to a wide range of bioactive molecules associated with cellular signaling and adhesion. It is involved in regulation of proliferation, differentiation, migration, and adhesion of different types of cells in health and disease. The endocan concentration is related to endothelial activation and neovascularization [47]. Endocan levels are elevated in

Novel biomarkers	System/cells	Effect
Metabolic/biochemical		
ADMA	Inhibitor NOS	Endothelial dysfunction
MMP2, MMP9	Intercellular matrix	Intracellular matrix rearrangement
TIMP2, TIMP9		
Myeloperoxidase (MPO)	Activated neutrophils and macrophages	Production of oxidative stress
ox-LDL, 8-hydroxy-2′-deoxygunosine.	Lipids, activated proteins	Reactive oxygen species and products (with increased oxidative stress
MDA (lipid peroxidation), protein carbonyl (PCO)		
IL-6, TNF-alfa	lymphocytes	Proinflammatory cytokines
Toll-like receptor 4	lymphocytes	Innate immunity
NO metabolites (nitrates, nitrites)	Endothelium (NO)	Vasodilation (NO) and nitrosylation
Functional/structural		
Flow-mediated dilation (FMD)	Blood vessels (endothelial function)	NO dependent, or COX, EDHF, EDCF dependent
Intima-media thickness (IMT)	Blood vessels (endothelial function + VSMC)	multifactorial

Table 1. Potential novel biomarkers of atherosclerosis.

conditions such as tumor progression, hypertension, chronic kidney disease, and renal transplant rejection [48]. Tadzic et al. [49] have described an increased expression of cell adhesion molecules, intracellular adhesion molecule's (ICAM) and vascular cell adhesion molecule's (VACM) ligands, together with decrease of sCAMs and endocan in hypertensive patients on amlodipine therapy with reduction in blood pressure, suggesting de-activation of endothelium. Systolic and diastolic blood pressure was positively correlated with ICAM-1 and VCAM-1, and systolic blood pressure was negatively correlated with CD11a/LFA-1. Endocan significantly positively correlated with ICAM-1 [49].

Diabetes is associated with increased circulating levels of endothelium-derived adhesion molecules and plasminogen activator inhibitor-1, which have pro-inflammatory and prothrombotic effects [50, 51]. In endothelial dysfunction, the endothelium can express adhesion molecules responsible for the withdrawal of leukocytes from vascular wall, such as VCAM-1 and ICAM-1 [36]. Also, E-selectin and platelet endothelial cell adhesion molecule have been expressed in atherosclerotic lesions and are involved in mononuclear cell adhesion to the vascular endothelium [52, 53]. The main difference in the activation of adhesion molecules is that the expression of ICAM-1 increases after cell activation, while E-selectin and VCAM-1 are only induced after cell activation. It is demonstrated that hyperglycemia results in the expression of adhesion molecules: endothelial-leukocyte adhesion molecule-1, VCAM-1, and ICAM-1 in human vascular endothelial cells [54]. In the rat mesenteric microcirculation, only intraperitoneal co-administration of IL-1β with D-glucose increased leukocyte rolling flux,

adhesion, and migration, indicating that pro-inflammatory environment in diabetes is a critical factor in pro-atherosclerotic effects of hyperglycemia [54, 55].

Increased concentration of plasma glucose activates the endothelium [56–58]. Exposure of arterial tissue to increased glucose level induces superoxide production and impairs NO bioavailability in the vascular wall which leads to increased oxidative stress in these conditions [59]. In diabetes mellitus, the production of superoxide and NADPH oxidase activity are increased [60, 61] which promote activation of the pro-inflammatory transcription factor NFκB [56]. The transcription factor NFκB is one of key regulator of endothelial activation and is included in insulin resistance [62, 63]. This is supported by study in obese persons [64]. Salsalate (an anti-inflammatory drug) increased expression of the inhibitor of NF-κB and reduced NFκB activation in freshly isolated endothelial cells taken from obese persons. Salsalate increased brachial artery flow-mediated dilation and reduced nitrotyrosine and expression of NADPH oxidase p47(phox) in these endothelial cells [64]. **Table 1** presents some of the proposed novel biomarkers for atherosclerosis, which could also be related to other cardiometabolic diseases.

3. Functional biomarkers of vascular (endothelial) function

3.1. Assessment of microvascular endothelial function

3.1.1. Coronary microvascular function assessment

In the past, coronary angiography (of larger conductance arteries, i.e., coronary vessels) was considered a gold standard for evaluation of the severity and extent of CAD. However, in the last two decades, the attention was shifted to the coronary microcirculation as the possible site of anatomical and functional abnormalities crucial for the development and progression of final myocardial ischemia. Thus, functional assessment of coronary microcirculation and its endothelial function became a challenge. For a long time, measurement of changes in coronary blood flow (CBF) during coronary angiography (Doppler wires) has been used as a surrogate parameter for coronary microvascular function assessment [65]. The final result of this measurement is assessment of coronary flow reserve (CFR) which presents the ratio between the maximal CBF during maximal coronary hyperemia (provoked by adenosine infusion, pacing, or exercise) and the resting CBF. It has been demonstrated that CFR is both endothelium-dependent and endothelium-independent, and CFR below 2.0 is considered abnormal [66]. For coronary microvascular endothelium-dependent vasodilation assessment, instead of maximal CBF, CBF in response to endothelium-dependent vasodilator (commonly ACh) infused at increasing concentrations is calculated. Another method for the assessment of coronary microvascular function includes the measurement of the number of cineangiographic frames that it takes to fill a distal vessel with proximal injection of contrast. This method is named Thrombolysis in Myocardial Infarction (TIMI) and provides semi-quantitative assessment of epicardial coronary blood flow [67]. The main advantage of the abovementioned methods is to measure microvascular endothelial function directly in this clinically important

vascular bed. However, main limitations are the cost, invasive nature, and therefore a limited population in which these measurements can be actually performed (symptomatic individuals requiring invasive coronary angiography) [68].

In recent years, a number of other methods have been developed among them: (a) blood oxygen level-dependent (BOLD) magnetic resonance imaging (MRI), a functional test that detects a dissociation of tissue hemoglobin from blood flow, is shown to be a useful tool for coronary endothelial function assessment [69]; (b) positron-emission tomography (PET) myocardial perfusion imaging that is based on the assessment of regional myocardial blood flow both at rest and during various forms of vasomotor stress [70] and presents a powerful tool to evaluate the effects of CV risk factors on the health of the microvasculature and its endothelium [71]; and (c) myocardial perfusion echocardiography, a bedside method with relatively low cost that is capable to detect myocardial perfusion abnormalities and quantify regional and global coronary blood flow [72]. Despite the fact that these new methods manage to provide noninvasive evaluation of coronary microvasculature directly at the site, they are still unacceptable for routine screening due to their limited availability, expensive equipment and associated costs, and lack of experienced/trained staff. Considering that endothelial function is a systemic disorder, peripheral vascular beds and their microcirculation present a good alternative that provides an easier access and need less elaborate equipment.

3.1.2. Venous occlusion plethysmography

Venous occlusion plethysmography presents a semi-invasive technique (arterial puncture) for assessment of forearm blood flow (and the corresponding microcirculation) changes before and after infusion of vasoactive substances into a cannulated brachial artery [3]. The method was introduced 90 years ago by Hewlett and van Zwaluwenburg [73], and the basic methodology has changed little since its first description. Basic principle of this method is to stop the return of venous blood from the forearm (inflating the cuff over the diastolic pressure value) with the preserved arterial blood inflow to the forearm, leading to a linear increase in blood flow at a given time, which is proportional to the arterial blood inflow. Another cuff excludes the blood flow through the hand to reduce the temperature fluctuations of the blood flow depending on the temperature. Changes in the flow are recorded by changing the electrical resistance of the plethysmograph located around the longest part of the forearm [74]. The main advantage of this method is that it provides assessment of endothelium-dependent and -independent vasodilation and mechanisms mediating it by intra-arterial infusion of vasoactive substances (e.g., ACh or sodium nitroprusside, and SNP), hormones, and drugs. However, its important limitation is that it could not strictly discern between macro- and microcirculation. Final results are expressed as ratio between blood flow changes in both arms and are well reproducible [75]. Regarding the mechanisms, some studies reported that ACh-induced dilation was inhibited by a NOS inhibitor, L-N^G-monomethyl Arginine citrate (L-NMMA) [76], suggesting that NO is the main vasodilator mediating endothelium-dependent vasodilation in this vascular bed. On the other hand, others reported that EDHF has a crucial role in mediating microvascular endothelial-dependent vasodilation, especially in population with multiple CV risk factors [77]. A large number of studies used venous occlusion plethysmography to assess the association between endothelial dysfunction and CV risk

factors, and described it in hypercholesterolemia [78], diabetes mellitus [79], cigarette smoking [80], and aging [81], while the results in hypertensive patients were conflicting [3, 4, 82, 83]. Even though the method and pharmacologically induced vasodilation provide an insight into peripheral microvascular patho(physiology), venous occlusion plethysmography is characterized by several limitations and disadvantages, including its semi-invasive character, limited comparison between groups due to different initial blood pressure and forearm blood flow, different sizes of the forearm, etc., [68].

3.1.3. Reactive hyperemia peripheral arterial tonometry (RH-PAT)

RH-PAT is a noninvasive technique designed for assessment of peripheral microvascular function. This method reflects changes in finger pulse volume amplitude during reactive hyperemia (an equivalent to finger plethysmography). PAT device includes digital probes that are placed on the tip of each index finger and a blood pressure cuff (for provoking occlusion) that is placed around the upper arm of the study arm, while the other arm serves as a control [84, 85]. Vascular occlusion is provoked by inflation of the blood pressure cuff to a 50 mmHg above systolic blood pressure for 5 min. The PAT signal is recorded 10 min prior occlusion, and for 10 min after the cuff is deflated. The final result of this measurement is calculated as the ratio of average amplitude of the PAT signal over a period of 1 min, starting 1 min after cuff deflation to average amplitude of the PAT signal for 3 min at baseline (RH-PAT index) that is normalized to the control arm [84, 85]. Studies have shown that RH-PAT is at least partly NO dependent. Importantly, studies by Rubinshtein et al. and Akiyama et al. reported that RH-PAT may be a useful tool for prediction of future CV events in patients with CV risk [86, 87]. Advantages of this method are that it is noninvasive, it is very simple and reproducible, and that it is operator independent (RH-PAT index is measured automatically). Even though RH-PAT is very similar to FMD of the brachial artery, Framingham Heart Study has revealed that there was no significant correlation between RH-PAT and FMD [88]. Moreover, the same study reported that different CV risk factors contribute differently to changes in FMD and RH-PAT [89], suggesting that these two methods assess different vascular beds, and that macro- and microvascular endothelium is differently susceptible to various risk factors.

3.1.4. Laser Doppler (LD) flowmetry

Because of its easy accessibility, the skin presents an appropriate site to study peripheral microcirculation, which was proposed as a suitable marker of systemic microvascular function in various diseases [89]. Therefore, in recent years, a number of simple and noninvasive methods have been developed in order to assess peripheral microcirculation. Still, it is an open question whether skin microcirculation is actually a representative indicator of the microvascular function of other organs. Despite that skin microvascular function was extensively used over the past 30 years to investigate vascular mechanisms in various diseases including hypertension [90, 91], obesity [92], diabetes [93, 94], aging, kidney disease [95], etc.

The laser Doppler (LD) technique is based on the estimation of the flow rate in the skin microcirculation using the laser beam reflection from the erythrocyte in microcirculation and its wavelength change (Doppler's effect) [96]. Computer software determines the flow

size, which is rather an index of skin perfusion (flux) than direct measure of skin blood flow. Results are commonly expressed in arbitrary units (perfusion units, PU) or as cutaneous vascular conductance (CVC; flux divided by arterial pressure in mV/mmHg) [96]. The first developed technique was the laser Doppler flowmetry (LDF) that measures blood flow in a single point and thus over a small volume but with a high sampling frequency. A major limitation of this technique is its spatial variability, due to regional heterogeneity of skin perfusion and blood flow measurement in a single point [97]. Later, laser Doppler imaging (LDI) was developed, which provides a 2D image of skin microvascular perfusion using the same principle as LDF. Since this method assess flow over larger surface than LDF, it managed to reduce spatial variability, but it appears to be much slower than LDF, making rapid changes in blood flow difficult to record [98]. Both techniques are commonly used for microvascular reactivity assessment in response to various stimuli, including iontophoresis of vasoactive drugs, post-occlusive reactive hyperemia (PORH), and thermal challenges [98].

Microdialysis is a technique based on intradermal insertion of small fibers for continuous delivery of drugs into a small area of tissue. This type of drug delivery provides avoiding its systemic effect [99] and it provides controlled drug application and absence of current-induced vasodilation, compared to iontophoresis. However, microdyalisis is invasive and painful, and justifies the use of local anesthesia which might also affect the blood flow and thus impact the results. It was commonly used to assess the role of NO in PORH and the thermal hyperemia response of skin microcirculation measured with LDF [98].

Iontophoresis is a method for noninvasive transdermal drug delivery (charged molecules) using low-density electric current. ACh and SNP iontophoresis are widely used for assessment of endothelium-dependent and endothelium-independent vasodilation of skin microcirculation [98, 100]. Regarding endothelium-dependent dilation, studies reported that ACh-induced dilation seems to be predominantly mediated by COX metabolites (although results are still conflicting) [101, 102], and NO does not extensively contribute to such dilation [103] in skin microcirculation. Beside endothelial-dependent vasodilation, ACh administration induces neural axon reflex-mediated dilation as well [104]. Iontophoresis is associated with several issues: (a) current itself may induce nonspecific vasodilation, which could interfere with the vasodilation potency of administrated drug, and it was suggested that it depends on the delivered electrical charge and the current delivery pattern [105]; (b) current-induced dilation also may depend on vehicles that have been used to dilute drugs (e.g., tap water, distilled water, deionized water, and saline), but this was not observed for ACh and SNP [106]; (c) skin resistance may influence drug delivery, and thus reduce skin resistance which was suggested as a part of good practice [100]; (d) spatial variability of ACh and SNP, suggesting that monitoring larger areas using LDI, rather than LDF provides better reproducibility [107, 108]; and (e) site of iontophoresis, since for example SNP-induced dilation could not be provoked on finger pulp, but it was provoked on the dorsum of the finger [109]. To summarize, ACh and SNP iontophoresis is widely used for endothelium-dependent and -independent microvascular vasodilation assessment in both healthy and various diseases. However, when interpreting results, complexity of mechanisms involved in these responses should be taken into account. Moreover, studies using iontophoresis should be carefully designed to reduce non-specific current-induced dilation by using low intensity current; saline should be rather

used as vehicle than distilled water; pre-treatment with anesthetic should be considered; and, finally, skin resistance should be reduced as much as possible.

PORH refers to an increase in (micro)vascular blood flow due to transient short vascular occlusion, and represents a test that is commonly used for assessment of microvascular reactivity [98]. According to the literature, several mechanisms are involved in microvascular PORH response, including sensory nerves involvement via neural axon reflex [110], metabolic and myogenic component, and endothelial-dependent vasodilators production. Regarding endothelium, EDHF was suggested as an important mediator of PORH [9], while the role of prostaglandins is still not clarified [11, 12]. Studies have reported that eNOS inhibition does not alter PORH, suggesting that NO is not normally involved in forearm microvascular PORH [111]. It has been suggested that inhibition of COX inhibition may unmask the NO dependence of PORH in human cutaneous circulation [12]. Despite an evident role of endothelium-derived vasoactive mediators in skin microvascular PORH, it should be used as a tool for assessment of general microvascular reactivity, rather than a measure for microvascular endothelial function [89]. PORH can be used in conjunction with both LDF and LDI, but an advantage is given to the LDF, because LDI is considered too slow to track microvascular kinetics during PORH. Moreover, inter-day reproducibility of single-point LDF was excellent when the probe was placed on exactly the same site from one day to another [112]. While recording skin microvascular PORH homogenizing both skin and room temperature is important, since temperature plays a key role in regulation of baseline flux [97]. Another issue is related to the PORH measurement, and that is heterogeneity in study design, especially vascular occlusion duration (from 1 to 15 min) [113] and different cuff pressures used, ranging between 160 and 220 mmHg [114]. Although it is accepted as a good tool for microvascular reactivity assessment, this method still requires standardization.

Local thermal hyperemia (LTH) presents peripheral skin microvascular response to local heating mediated by joint effect of neural-dependent and NO-dependent vasodilator pathway [98]. LTH is characterized by initial peak (within the first 5 min) which depends on sensory nerves, and by sustained plateau which is mostly NO-dependent [115]. LTH has better reproducibility in conjunction with LDI, rather than a single-point LDF, and this reproducibility depends on the site of measurement too [97]. Similar to PORH, there is heterogeneity in the study design using LTH, including local warming temperature (42–43°C) [116], the time of heating, and the nature of the device used to heat the skin [89]. Another used thermal stimulus is local cooling that induces an initial vasoconstriction followed by transient vasodilation, and finally, prolonged vasoconstriction [116]. It has been demonstrated that initial vasoconstriction depends on norepinephrine, and prolonged vasoconstriction involved both norepinephrine and inhibition of NO system [116]. Results have shown that this method has the best reproducibility when the cooling protocol lasts for 30 min at 15°C [97].

Laser speckle contrast imaging is a novel technique that combines advantages of LDF and LDI, with very good inter-day reproducibility for both PORH and LTH measurements [117, 118]. This method is based on speckle contrast analysis that provides an index of blood blow. A potential limitation of this technique is its sensitivity to movements and potential challenging data analysis, but despite limitations, this method is expected to be a remarkable tool for microvascular function assessment, especially when coupled with PORH and/or LTH [89].

3.1.5. Fingertip digital thermal monitoring (DTM)

Fingertip digital thermal monitoring (DMT) of vascular reactivity represents a noninvasive, reproducible, operator-independent technique based on changes in fingertip temperature during cuff-occlusive reactive hyperemia [119]. This method relies on a premise that changes in fingertip temperature during and after vascular occlusion that reflects changes in blood flow and thus microvascular and endothelial function [120]. So far, studies have reported that vascular function measured by DTM correlate with Framingham Risk Score and coronary artery calcium score (a measurement of the amount of calcium in the walls of the coronary arteries using a special computed tomography (CT) scan of heart) independently of age, sex, and traditional cardiac risk factors [121]. Although clinical implications of DTM are promising, more studies on the mechanisms mediating this vascular response and large prospective trials are needed to establish the real research and clinical value of this method.

3.2. Assessment of macrovascular function

3.2.1. Flow-mediated dilation

FMD of the brachial artery is the most widely used noninvasive *in vivo* method for an indirect assessment of endothelial function of conduit vessels introduced by Celermajer and colleagues [5]. It provides decisive information about the ability of the endothelium to respond to particular stimulus (reactive hyperemia). In this method, an arterial occlusion cuff is placed to the forearm and inflated to stop the anterograde blood flow, thus generating ischemia. Consequently, distal from that the occlusion, in the resistance arteries, vasodilation occurs, and when the sphygmomanometer is deflated, reactive hyperemia occurs in the brachial artery. The method involves ultrasound arterial imaging in two conditions, at rest (baseline) and during reactive hyperemia after 5 min arterial occlusion, and FMD is expressed as the % difference between that two measured diameters [122]. The exact mechanism mediating FMD during reactive hyperemia has not been fully elucidated; it is considered that shear stress-induced NO is the main mediator [76, 85], but also other endothelium-derived vasodilator factors may also contribute [123]. Because reactive hyperemia flow, induces increased shear stress on endothelium challenges FMD, it might be a significant measure of peripheral microvascular function because reactive hyperemia is greatly dependent on maximal forearm resistance [124]. Furthermore, peripheral endothelial function as assessed by FMD correlates with vascular function of coronary artery [125]. In addition, impaired FMD is one of the early manifestations of vascular disease, and may be an important indicator of endothelium injury [126].

However, although the principle of this technique seems simple, its application is technically challenging and requires comprehensive practicing and standardization [127, 128]. Easy access of this noninvasive method is one of the main advantages of this method, while other advantages being a good correlation with invasive epicardial vascular function assessment, possibility to assess other important parameters (i.e., flow, baseline arterial diameters and flow-mediated constriction), and low costs [68].

To ensure that impaired FMD is not due to underlying vascular smooth muscle dysfunction or alterations in vascular structure but truly a consequence of endothelial dysfunction,

response to nitroglycerine is used [127, 129, 130]. Nitroglycerine-induced vasodilation was significantly reduced in patients with cardiovascular disease [129]. Additionally, nitroglycerine-induced vasodilation was impaired in patients with atherosclerosis [131]. FMD should be interpreted as an index of vascular function reflecting both endothelium-dependent and -independent vasodilation in individuals with impaired nitroglycerine-induced vasodilation [129]. Furthermore, coronary artery dilation in response to nitroglycerine is impaired in patients with coronary heart disease which predicts long-term atherosclerotic disease progression and cardiovascular event rate [132]. These findings suggest that nitroglycerine-induced vasodilation *per se* may be a marker of the grade of atherosclerosis and predictor of cardiovascular events. However, the relationship between nitroglycerine-induced vasodilation and the risk for future cardiovascular events should still be established.

3.2.2. New method for assessment of endothelial function—measurement of ezFMD

Since FMD requires an expensive ultrasound system and high levels of technical skills, a novel method for measurement of endothelial function, namely, measurement of enclosed-zone flow-mediated dilatation (ezFMD) was developed [133]. ezFMD is a noninvasive method which assesses the level of vasodilatation from the oscillation signals transmitted to a sphygmomanometer cuff attached to the upper arm. In patients with cardiovascular diseases, ezFMD was significantly lower than in age- and gender-matched healthy individuals. In addition, cardiovascular risk factors were independent predictors of ezFMD. ezFMD was significantly correlated with conventional FMD [134]. Conventional measurement of FMD by ultrasound is measured by the change in vascular diameter, whereas ezFMD is based on the change in vascular volume. Both methods are equally valuable for assessing endothelial function, however, measurement of ezFMD is easier and less biased than measurement of FMD.

3.2.3. Coronary epicardial vasoreactivity

Quantitative coronary angiography (QCA) or intravascular ultrasound are methods for imaging vasomotor responses of epicardial coronary arteries, which enable tracing of changes in vessel diameters in response to endothelium-dependent interventions, e.g., intracoronary infusion of drugs or substances, such as acetylcholine [2]. Vessels with an intact endothelium vasodilate in response to ACh infusion, whereas segments with dysfunctional endothelial cells display abnormal vascular response [2]. Estimation of coronary endothelial function with intracoronary ACh provides diagnostic and prognostic data in patients with suspected coronary microvascular dysfunction.

Some of advantages of this method is direct assessment of the coronary vascular bed and represents gold standard for assessment of epicardial macrovasculature, while its disadvantage is invasiveness and limitation to those patients undergoing coronary angiography [68].

Physiologically, endothelium-dependent vasodilation occurs in response to exercise or tachycardia as a replacement for exercise, but also pacing induced tachycardia, and leads to increased flow-mediated endothelium-dependent vasomotion of the epicardial vessels that is impaired in atherosclerosis [68]. In healthy isolated intramyocardial porcine coronary

resistance arteries, bradykinin, serotonin, and the alpha 2-adrenergic agonist clonidine evoked endothelium-dependent relaxations, which were fully (clonidine) or partially (serotonin) mediated by NO, while vasodilator response to bradykinin seems to be mediated by some other endothelium-derived mediator, different from NO [135]. Further, cold pressor test (CPT), in which the subject puts his hand into ice water, is another mode to assess epicardial vasoreactivity. In the study by Nabel et al., the response to CPT was assessed in patients with angiographically normal coronary arteries, in patients with mild coronary atherosclerosis and in patients with advanced coronary stenosis, using quantitative angiography and Doppler flow velocity measurements. Normal vessels exhibited vasodilation (partly related to beta-adrenergic receptor stimulation and partly due to flow-mediated dilation or alpha-2 adrenergic receptor activation) while atherosclerotic vessels exhibited vasoconstriction in response to CPT, possibly due to altered sensitivity to adrenergic stimulation and/or some other impairment of endothelium-dependent vasodilation [136].

3.2.4. Pulse wave velocity

Pulse wave velocity (PWV) is the velocity at which the pulse pressure wave spreads from the left ventricle (at the end of ventricular ejection) to the periphery. It results in an earlier return of the reflected wave which increases the pressure and subsequently the afterload of the left ventricle and reduces coronary artery perfusion pressure during diastole. One of the most frequently used noninvasive methods for the assessment of aortic stiffness is carotid-femoral (aortic) PWV [137]. It is a simple, noninvasive, and reproducible method which has been used as a gold standard and provides a predictive value of aortic stiffness for future cardiovascular events [138]. PWV has been used as significant marker of cardiovascular risk. Data indicate that increased arterial stiffness is being independently predictive of coronary artery disease, stroke, and cardiovascular events in general [139]. While PWV values are lower in healthy young individuals, the values of PWV increase with reduction of arterial elasticity [140].

Applanation tonometry is another method that is used for pulse wave analysis. Rather than directly assessing aortic pulse wave, it estimates aortic pulse wave from the common carotid artery or the radial artery pulse waves. As the measurement is easier, radial artery tonometry has been the most commonly recommended approach [137]. Since the method can detect changes that might be related to vascular health, even before the onset of signs and symptoms, yet, the PWV analysis occupies an important place in clinical practice [141]. This method has some limitations that are related to associated comorbidities, such as metabolic syndrome, obesity, and diabetes, because the femoral pressure waveform may be difficult to record accurately in these patients [137].

3.2.5. Intima-media thickness

Carotid intima-media thickness (CIMT) is a method that evaluates extra-cranial carotid arteries by high-resolution ultrasound, and represents an important marker of subclinical atherosclerosis [142]. CIMT is increased in atherosclerosis and also correlates with

coronary artery disease [143] and cerebrovascular disease [144]. CIMT represents the combined width of the intima and media; in healthy individuals, it is composed almost entirely of media, with a progressive intimal thickening or hypertrophy of media, determined by age, gender, and hypertension [145]. The major advantage of CIMT is that it is noninvasive and reproducible, relatively inexpensive to perform, also widely available and well standardized [146].

3.2.6. Functional endothelial biomarkers in cardiovascular diseases

The baseline pathogenic process in cardiovascular diseases, such as atherosclerosis and coronary artery disease, is an endothelial dysfunction with complex underlying mechanisms: oxidative stress, diminished vasoreactivity, hemostatic disturbances, and inflammation leading to the disease progression by modulating the arterial wall, promoting lipoprotein retention, plaque formation and possibly its destabilization. Endothelial dysfunction is characterized by endothelial dysfunction, impaired vascular homeostasis and reduced "anti"-mechanisms (-oxidant, -inflammatory, -thrombotic) and activated "pro"-mechanisms. Diagnostic tools for detecting endothelial dysfunction in humans are limited. They should be safe, cost-effective, noninvasive, repeatable, reproducible, and standardized. Current diagnostic methods are FMD, forearm plethysmography, finger-pulse plethysmography, PWV analysis, and coronary angiography. However, there is a need for additional diagnostic tools, biomarkers. For everyday clinical use, more and larger human-based studies are necessary to validate clinical usefulness of biomarkers [147, 148].

4. Conclusion

To find a specific and sensitive biomarker for any disease sometimes looks like a search for the Holy Grail—something precious but impossible to find. The reason for that could be those cardiometabolic diseases, all having a common point—endothelial dysfunction and it is likely that they have common underlying mechanisms leading to endothelial dysfunction. These mechanisms may be redundant and not activated at the same time and the same order, but certainly end up with impaired endothelium, and inappropriate vascular response to physiological stimuli with inability to compensate for pathophysiological events, finally leading to manifested disease and organ damage. One can only take with "a grain of salt" as many different biomarkers as possible and build up a picture of their relationship to the disease's etiopathogenesis, development, and prognosis.

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