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The Markers of Endothelial Activation

Ines Drenjancevic, Ivana Jukic, Ana Stupin,
Anita Cosic, Marko Stupin and
Kristina Selthofer-Relatic

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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. MDA (lipid peroxidation), protein carbonyl (PCO)	Lipids, activated proteins	Reactive oxygen species and products (with increased oxidative stress
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 pro-thrombotic 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.

Acknowledgements

Author details

Ines Drenjancevic[1,4]*, Ivana Jukic[1,2,4], Ana Stupin[1,2,4], Anita Cosic[1,4], Marko Stupin[1,3,4] and Kristina Selthofer-Relatic[1,3,4]

*Address all correspondence to: ines.drenjancevic@mefos.hr

1 Faculty of Medicine, University of Osijek, Osijek, Croatia

2 Faculty of Dental Medicine and Health Studies, University of Osijek, Osijek, Croatia

3 Internal Clinic, Clinical Hospital Center, Osijek, Croatia

4 Croatian National Scientific Center of Excellence for Personalized Health Care, University of Josip Juraj Strossmayer, Osijek, Croatia

References

[1] Flammer AJ, Luscher TF. Three decades of endothelium research: From the detection of nitric oxide to the everyday implementation of endothelial function measurements in cardiovascular diseases. Swiss Medical Weekly. 2010;**140**:w13122

[2] Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, Ganz P. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. The New England Journal of Medicine. 1986 Oct 23;**315**(17):1046-1051

[3] Linder L, Kiowski W, Bühler FR, Lüscher TF. Indirect evidence for release of endothelium-derived relaxing factor in human forearm circulation in vivo: Blunted response in essential hypertension. Circulation. 1990;**81**:1762-1767

[4] Panza JA, Quyyumi AA, Brush JE Jr, Epstein SE. Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. The New England Journal of Medicine. 1990;**323**(1):22-27

[5] Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, Deanfield JE. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet. 1992;**340**:1111-1115

[6] Anderson TJ, Gerhard MD, Meredith IT, Charbonneau F, Delagrange D, Creager MA, Selwyn AP, Ganz P. Systemic nature of endothelial dysfunction in atherosclerosis. The American Journal of Cardiology. 1995;**75**:71B-74B

[7] Brunner H, Cockcroft JR, Deanfield J, Donald A, Ferrannini E, Halcox J, Kiowski W, Luscher TF, Mancia G, Natali A, Oliver JJ, Pessina AC, Rizzoni D, Rossi GP, Salvetti A, Spieker LE, Taddei S, Webb DJ. Endothelial function and dysfunction, part II: Association with cardiovascular risk factors and diseases: A statement by the working group on Endothelins and endothelial factors of the European Society of Hypertension. Journal of Hypertension. 2005;**23**:233-246

[8] Trochu JN, Bouhour JB, Kaley G, Hintze TH. Role of endothelium-derived nitric oxide in the regulation of cardiac oxygen metabolism: Implications in health and disease. Circulation Research. 2000;**87**:1108-1117

[9] Lorenzo S, Minson CT. Human cutaneous reactive hyperaemia: Role of BKCa channels and sensory nerves. The Journal of Physiology. 2007;**585**:295-303

[10] Binggeli C, Spieker LE, Corti R, Sudano I, Stojanovic V, Hayoz D, Luscher TF, Noll G. Statins enhance postischemic hyperemia in the skin circulation of hypercholesterolemic patients: A monitoring test of endothelial dysfunction for clinical practice? Journal of the American College of Cardiology. 2003;**42**:71-77

[11] Dalle-Ave A, Kubli S, Golay S, Delachaux A, Liaudet L, Waeber B, Feihl F. Acetylcholine-induced vasodilation and reactive hyperemia are not affected by acute cyclo-oxygenase inhibition in human skin. Microcirculation. 2004;**11**:327-336

[12] Medow MS, Taneja SJM. Cyclooxygenase and nitric oxide synthase dependence of cutaneous reactive hyperemia in humans. American Journal of Physiology-Heart and Circulatory Physiology. 2007;**293**:H425-H432

[13] Lavi S, Prasad A, Yang EH, Mathew V, Simari RD, Rihal CS, Lerman LO, Lerman A. Smoking is associated with epicardial coronary endothelial dysfunction and elevated white blood cell count in patients with chest pain and early coronary artery disease. Circulation. 2007;**115**(20):2621-2627

[14] Magness RR, Shideman CR, Habermehl DA, Sullivan JA, Bird IM. Endothelial vasodilator production by uterine and systemic arteries. V. Effects of ovariectomy, the ovarian cycle, and pregnancy on prostacyclin synthase expression. Prostaglandins & Other Lipid Mediators. 2000;**60**(4-6):103-118

[15] Lewis PJ, Boylan P, Friedman LA, Hensby CN, Downing I. Prostacyclin in pregnancy. British Medical Journal. 1980;**280**:1581-1582

[16] Morton JS, Davidge ST. Arterial endothelium-derived hyperpolarization: Potential role in pregnancy adaptations and complications. Journal of Cardiovascular Pharmacology. 2013;**61**:197-203

[17] Juonala M, Viikari JSA, Alfthan G, Marniemi J, Kähönen M, Taittonen L, Laitinen T, Raitakari OT. Brachial artery flow-mediated dilation and asymmetrical Dimethylarginine in the cardiovascular risk in young Finns study. Circulation. 2007;**116**:1367-1373

[18] Rizos D, Eleftheriades M, Batakis E, Rizou M, Haliassos A, Hassioakos D, Botsis D. Levels of asymmetric dimethylarginine throughout normal pregnancy and in pregnancies complicated with preeclampsia or had small for gestational age baby. The Journal of Maternal-Fetal and Neonatal Medicine. 2012;**25**(8):1311-1315

[19] Coussons-Read ME, Lobel M, Carey JC, Kreither MO, D'Anna K, Argys L, Ross RG, Brandt C, Cole S. The occurrence of preterm delivery is linked to pregnancy-specific distress and elevated inflammatory markers across gestation. Brain, Behavior, and Immunity. 2012;**26**(4):650-659

[20] Himburg HA, Dowd SE, Friedman MH. Frequency-dependent response of the vascular endothelium to pulsatile shear stress. American Journal of Physiology. Heart and Circulatory Physiology. 2007;**293**:H645-H653

[21] Nishida K, Harrison DG, Navas JP, Fisher AA, Dockery SP, Uematsu M, Nerem RM, Alexander RW, Murphy TJ. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. The Journal of Clinical Investigation. 1992;**90**:2092-2096

[22] Uematsu M, Ohara Y, Navas JP, Nishida K, Murphy TJ, Alexander RW, Nerem RM, Harrison DG. Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. American Journal of Physiology. Cell Physiology. 1995;**269**:C1371-C1378

[23] Shin HY, Gerritsen ME, Bizios R. Regulation of endothelial cell proliferation and apoptosis by cyclic pressure. Annals of Biomedical Engineering. 2002;**30**:297-304

[24] Cheng JJ, Wung BS, Chao YJ, Wang DL. Cyclic strain-induced reactive oxygen species involved in ICAM-1 gene induction in endothelial cells. Hypertension. 1998;**31**:125-130

[25] Gradinaru D, Borsa C, Ionescu C, Prada GI. Oxidized LDL and NO synthesis-biomarkers of endothelial dysfunction and ageing. Mechanisms of Ageing and Development. 2015;**151**:101-113

[26] Mehta JL. The role of LOX-1, a novel lectin-like receptor for oxidized low density lipoprotein, in atherosclerosis. The Canadian Journal of Cardiology. 2004 Aug;**20** (Suppl B):32B-36B

[27] Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. Lancet. 1992 Mar 7; **339**(8793):572-575

[28] Stühlinger MC, Oka RK, Graf EE, Schmölzer I, Upson BM, Kapoor O, Szuba A, Malinow MR, Wascher TC, Pachinger O, Cooke JP. Endothelial dysfunction induced by hyperhomocyst(e)inemia: Role of asymmetric dimethylarginine. Circulation. 2003 Aug 26;**108**(8):933-938

[29] 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

[30] Cook S, Hugli O, Egli M, Ménard B, Thalmann S, Sartori C, Perrin C, Nicod P, Thorens B, Vollenweider P, Scherrer U, Burcelin R. Partial gene deletion of endothelial nitric oxide synthase predisposes to exaggerated high-fat diet-induced insulin resistance and arterial hypertension. Diabetes. 2004;**53**(8):2067-2072

[31] Asagami T, Abbasi F, Stuelinger M, Lamendola C, McLaughlin T, Cooke JP, Reaven GM, Tsao PS. Metformin treatment lowers asymmetric dimethylarginine concentrations in patients with type 2 diabetes. Metabolism. 2002;**51**(7):843-846

[32] Stühlinger MC, Abbasi F, Chu JW, Lamendola C, McLaughlin TL, Cooke JP, Reaven GM, Tsao PS. Relationship between insulin resistance and an endogenous nitric oxide synthase inhibitor. JAMA. 2002;**287**(11):1420-1426

[33] Falck-Hansen M, Kassiteridi C, Monaco C. Toll-like receptors in atherosclerosis. International Journal of Molecular Sciences. 2013;**14**(7):14008-14023

[34] Cole JE, Georgiou E, Monaco C. The expression and functions of toll-like receptors in atherosclerosis. Mediators of Inflammation. 2010;**2010**:393946

[35] Javed Q. Clinical implications of tumor necrosis factor-alpha, Interleukin-6 and resistin in coronary artery disease. World Journal of Cardiovascular Diseases. 2014;**4**:416-421

[36] Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation. 2002;**105**:1135-1143

[37] Takaishi H, Taniguchi T, Takahashi A, et al. High glucose accelerates MCP-1 production via p38 MAPK in vascular endothelial cells. Biochemical and Biophysical Research Communications. 2003;**305**:122-130

[38] Festa A, D'Agostino R Jr, Howard G, Mykkanen L, Tracy RP, Haffner SM. Chronic subclinical inflammation as part of the insulin resistance syndrome: The insulin resistance atherosclerosis study (IRAS). Circulation. 2000;**102**:42-47

[39] Dandona P, Weinstock R, Thusu K, Abdel-Rahman E, Aljada A, Wadden T. Tumor necrosis factoralpha in sera of obese patients: Fall with weight loss. The Journal of Clinical Endocrinology and Metabolism. 1998;**83**:2907-2910

[40] Vozarova B, Weyer C, Hanson K, Tataranni PA, Bogardus C, Pratley RE. Circulating interleukin-6 in relation to adiposity, insulin action, and insulin secretion. Obesity Research. 2001;**9**:414-417

[41] Schalkwijk CG, Poland DC, van Dijk W et al. Plasma concentration of C-reactive protein is increased in type I diabetic patients without clinical macroangiopathy and correlates with markers of endothelial dysfunction: Evidence for chronic inflammation. Diabetologia 1999;**42**:351-357

[42] Ji SR, Wu Y, Potempa LA, Liang YH, Zhao J. Effect of modified C-reactive protein on complement activation: A possible complement regulatory role of modified or monomeric C-reactive protein in atherosclerotic lesions. Arteriosclerosis, Thrombosis, and Vascular Biology. 2006;**26**(4):935-941

[43] Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. Journal of the American Medical Association. 2001;**286**(3):327-334

[44] Schulze MB, Rimm EB, Li T, Rifai N, Stampfer MJ, Hu FB. C-reactive protein and incident cardiovascular events among men with diabetes. Diabetes Care. 2004;**27**:889-894

[45] Mahajan K. Microparticles in atherosclerosis: Biomarkers of disease. J Clin Exp Cardiolog. 2015;**6**:1 http://dx.doi.org/10.4172/2155-9880.1000356

[46] Sarrazin S, Adam E, Lyon M, Depontieu F, Motte V, Landolfi C, Lortat-Jacob H, Bechard D, Lassalle P, Delehedde M. Endocan or endothelial cell specific molecule-1 (ESM-1): A potential novel endothelial cell marker and a new target for cancer therapy. Biochimica et Biophysica Acta. 2006 Jan;**1765**(1):25-37

[47] Kali A, Shetty KS. Endocan: A novel circulating proteoglycan. Indian Journal of Pharmacology. 2014 Nov-Dec;**46**(6):579-583

[48] Balta S, Mikhailidis DP, Demirkol S, Ozturk C, Celik T, Iyisoy A. Endocan: A novel inflammatory indicator in cardiovascular disease? Atherosclerosis. 2015 Nov;**243**(1):339-343

[49] Tadzic R, Mihalj M, Vcev A, Ennen J, Tadzic A, Drenjancevic I. The effects of arterial blood pressure reduction on endocan and soluble endothelial cell adhesion molecules (CAMs) and CAMs ligands expression in hypertensive patients on Ca-channel blocker therapy. Kidney & Blood Pressure Research. 2013;**37**(2-3):103-115

[50] Keaney JF Jr, Massaro JM, Larson MG, et al. Heritability and correlates of intercellular adhesion molecule-1 in the Framingham offspring study. Journal of the American College of Cardiology 2004;**44**:168-173

[51] Meigs JB, Mittleman MA, Nathan DM, et al. Hyperinsulinemia, hyperglycemia, and impaired hemostasis: The Framingham offspring study. Journal of the American Medical Association. 2000;**283**:221-228

[52] Maugeri N, Rovere-Querini P, Baldini M, et al. Translational mini-review series on immunology of vascular disease: Mechanisim of vascular inflammation and remodeling in systemic vasculitis. Clinical and Experimental Immunology. 2009;**156**:395-404

[53] Chavakis T, Bierhaus A, Nawroth PP. RAGE (receptor for advanced glycation end products): A central player in the inflammatory response. Microbes and Infection. 2004:1219-1225

[54] Altannavch TS, Roubalová K, Kučera P, et al. Effect of high glucose concentrations on expression of ELAM-1, VCAM-1 and ICAM-1 in HUVEC with and without cytokine activation. Physiological Research. 2004;**53**:77-82

[55] Azcutia V, Abu-Taha M, Romacho T, et al. Inflammation determines the pro-adhesive properties of high extracellular D-glucose in human endothelial cells in vitro and rat microvessels in vivo. PLoS One. 2010;**5**:e1009

[56] Maloney E, Sweet IR, Hockenbery DM, et al. Activation of NF-kappaB by palmitate in endothelial cells: A key role for NADPH oxidase-derived superoxide in response to TLR4 activation. Arteriosclerosis, Thrombosis, and Vascular Biology. 2009;**29**:1370-1375

[57] Pieper GM, Riaz uH. Activation of nuclear factor-kappaB in cultured endothelial cells by increased glucose concentration: Prevention by calphostin C. Journal of Cardiovascular Pharmacology. 1997;**30**:528-532

[58] Piga R, Naito Y, Kokura S, Handa O, Yoshikawa T. Short-term high glucose exposure induces monocyte-endothelial cells adhesion and transmigration by increasing VCAM-1 and MCP-1 expression in human aortic endothelial cells. Atherosclerosis. 2007;**193**:328-334

[59] TS1 L, Pei YH, Peng YP, Chen J, Jiang SS, Gong JB. Oscillating high glucose enhances oxidative stress and apoptosis in human coronary artery endothelial cells. Journal of Endocrinological Investigation. 2014 Jul;**37**(7):645-651. DOI: 10.1007/s40618-014-0086-5

[60] San Martin A, Du P, Dikalova A, et al. Reactive oxygen species-selective regulation of aortic inflammatory gene expression in type 2 diabetes. American Journal of Physiology. Heart and Circulatory Physiology. 2007;**292**:H2073-H2082

[61] Gao L, Mann GE. Vascular NAD(P)H oxidase activation in diabetes: A double-edged sword in redox signalling. Cardiovascular Research. 2009;**82**:9-20

[62] Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. The Journal of Clinical Investigation. 2006;**116**:1793-1801

[63] Read MA, Whitley MZ, Williams AJ, Collins T. NF-kappa B and I kappa B alpha: An inducible regulatory system in endothelial activation. The Journal of Experimental Medicine. 1994;**179**:503-512

[64] Pierce GL, Lesniewski LA, Lawson BR, Beske SD, Seals DR. Nuclear factor-{kappa}B activation contributes to vascular endothelial dysfunction via oxidative stress in overweight/obese middle aged and older humans. Circulation. 2009;**119**:1284-1292

[65] Beltrame JF, Crea F, Camici P. Advances in coronary microvascular dysfunction. Heart, Lung & Circulation. 2009;**18**:19-27

[66] Camici PG, Crea F. Coronary microvascular dysfunction. The New England Journal of Medicine. 2007;**356**:830-840

[67] Gibson CM, Cannon CP, Daley WL, Dodge JT, Jr, Alexander B, Jr, Marble SJ, McCabe CH, Raymond L, Fortin T, Poole WK, Braunwald E. TIMI frame count: A quantitative method of assessing coronary artery flow. Circulation 1996;**93**:879-888

[68] Flammer AJ, Anderson T, Celermajer DS, Creager MA, Deanfield J, Ganz P, Hamburg NM, Lüscher TF, Shechter M, Taddei S, Vita JA, Lerman A. The assessment of endothelial function: From research into clinical practice. Circulation. 2012;**126**(6):753-767

[69] Utz W, Jordan J, Niendorf T, Stoffels M, Luft FC, Dietz R, Friedrich MG. Blood oxygen level-dependent MRI of tissue oxygenation: Relation to endothelium-dependent and endothelium-independent blood flow changes. Arteriosclerosis, Thrombosis, and Vascular Biology. 2005;**25**:1408-1413

[70] Schindler TH, Schelbert HR, Quercioli A, Dilsizian V. Cardiac PET imaging for the detection and monitoring of coronary artery disease and microvascular health. JACC;**3**:623-640

[71] Al-Mallah MH, Sitek A, Moore SC, Di Carli M, Dorbala S. Assessment of myocardial perfusion and function with PET and PET/CT. Journal of Nuclear Cardiology. 2010;**17**(3):498-513

[72] Barletta G, Del Bene MR. Myocardial perfusion echocardiography and coronary microvascular dysfunction. World Journal of Cardiology. 2015;**7**(12):861-874

[73] Hewlett AW, van Zwaluwenburg JG. The rate of blood flow in the arm. Heart 1909;**1**:631-646

[74] Ian B. Wilkinson, David J Webb. Venous occlusion plethysmography in cardiovascular research: Methodology and clinical applications. British Journal of Clinical Pharmacology. 2001;**52**(6):631-646

[75] Petrie JR, Ueda S, Morris AD, Murray LS, Elliott HL, Connell JM. How reproducible is bilateral forearm plethysmography? British Journal of Clinical Pharmacology. 1998;**45**:131-139

[76] Joannides R, Haefeli WE, Linder L, Richard V, Bakkali EH, Thuillez C, Lüscher TF. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. Circulation. 1995;**91**:1314-1319

[77] Ozkor MA, Murrow JR, Rahman AM, Kavtaradze N, Lin J, Manatunga A, Quyyumi AA. Endothelium-derived hyperpolarizing factor determines resting and stimulated forearm vasodilator tone in health and in disease. Circulation. 2011;**123**:2244-2253

[78] Chowienczyk PJ, Watts GF, Cockcroft JR, Ritter JM. Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. Lancet. 1992;**340**:1430-1432

[79] Makimattila S, Liu ML, Vakkilainen J, et al. Impaired endothelium-dependent vasodilation in type 2 diabetes. Relation to LDL size, oxidized LDL, and antioxidants. Diabetes Care. 1999;**22**:973-981

[80] Heitzer T, Yla-Herttuala S, Luoma J, et al. Cigarette smoking potentiates endothelial dysfunction of forearm resistance vessels in patients with hypercholesterolemia: Role of oxidized LDL. Circulation. 1996;**93**:1346-1353

[81] Taddei S, Virdis A, Mattei P, et al. Aging and endothelial function in normotensive subjects and patients with essential hypertension. Circulation. 1995;**91**:1981-1987

[82] Panza JA, Casino PR, Kilcoyne CM, Quyyumi AA. Impaired endothelium-dependent vasodilation in patients with essential hypertension: Evidence that the abnormality is not at the muscarinic receptor level. Journal of the American College of Cardiology. 1994;**23**:1610-1616

[83] Bruning TA, Chang PC, Hendriks MGC, Vermeij P, Pfaffendorf M, Van Zwieten PA. In vivo characterization of muscarinic receptor subtypes that mediate vasodilatation in patients with essential hypertension. Hypertension. 1995;**26**:70-77

[84] Bonetti PO, Pumper GM, Higano ST, Holmes DR Jr, Kuvin JT, Lerman A. Noninasive identification of patients with early coronary atherosclerosis by assessment of digital reactive hyperemia. Journal of the American College of Cardiology. 2004;**44**:2137-2141

[85] Higashi Y. Assessment of endothelial function. History, methodological aspects, and clinical perspectives. International Heart Journal. 2015;**56**(2):125-134

[86] Rubinshtein R, Kuvin JT, Soffler M, et al. Assessment of endothelial function by non-invasive peripheral arterial tonometry predicts late cardiovascular adverse events. European Heart Journal. 2010;**31**:1142-1148

[87] Akiyama E, Sugiyama S, Matsuzawa Y, et al. Incremental prognostic significance of peripheral endothelial dysfunction in patients with heart failure with normal left ventricular ejection fraction. Journal of the American College of Cardiology. 2012;**60**:1778-1786

[88] Hamburg NM, Palmisano J, Larson MG, et al. Relation of brachial and digital measures of vascular function in the community: The Framingham heart study. Hypertension. 2011;**57**:390-396

[89] Roustit M, Cracowski JL. Non-invasive assessment of skin microvascular function in humans: An insight into methods. Microcirculation. 2012;**19**(1):47-64

[90] Antonios TF, Singer DR, Markandu ND, Mortimer PS, MacGregor GA. Structural skin capillary rarefaction in essential hypertension. Hypertension. 1999;**33**:998-1001

[91] Feihl F, Liaudet L, Waeber B, Levy BI. Hypertension: A disease of the microcirculation? Hypertension. 2006;**48**:1012-1017

[92] Levy BI, Schiffrin EL, Mourad JJ, Agostini D, Vicaut E, Safar ME, Struijker-Boudier HA. Impaired tissue perfusion: A pathology common to hypertension, obesity, and diabetes mellitus. Circulation. 2008;**118**:968-976

[93] Chang CH, Tsai RK, Wu WC, Kuo SL, Yu HS. Use of dynamic capillaroscopy for studying cutaneous microcirculation in patients with diabetes mellitus. Microvascular Research. 1997;**53**:121-127

[94] Yamamoto-Suganuma R, Aso Y. Relationship between post-occlusive forearm skin reactive hyperaemia and vascular disease in patients with type 2 diabetes – A novel index for detecting micro- and macrovascular dysfunction using laser Doppler flowmetry. Diabetic Medicine. 2009;**26**:83-88

[95] Kruger A, Stewart J, Sahityani R, O'Riordan E, Thompson C, Adler S, Garrick R, Vallance P, Goligorsky MS. Laser Doppler flowmetry detection of endothelial dysfunction in end-stage renal disease patients: Correlation with cardiovascular risk. Kidney International. 2006;**70**:157-164

[96] Stern MD. In vivo evaluation of microcirculation by coherent light scattering. Nature. 1975;**254**:56-58

[97] Roustit M, Blaise S, Millet C, Cracowski JL. Reproducibility and methodological issues of skin post-occlusive and thermal hyperemia assessed by single-point laser Doppler flowmetry. Microvascular Research. 2010;**79**:102-108

[98] Cracowski JL, Minson CT, Salvat-Melis M, Halliwill JR. Methodological issues in the assessment of skin microvascular endothelial function in humans. Trends in Pharmacological Sciences. 2006;**27**:503-508

[99] Clough GF. Microdialysis of large molecules. The AAPS Journal. 2005;**7**:E686-E692

[100] Turner J, Belch JJ, Khan F. Current concepts in assessment of microvascular endothelial function using laser Doppler imaging and iontophoresis. Trends in Cardiovascular Medicine. 2008;**18**:109-116

[101] Durand S, Tartas M, Bouye P, Koitka A, Saumet JL, Abraham P. Prostaglandins participate in the late phase of the vascular response to acetylcholine iontophoresis in humans. The Journal of Physiology. 2004;**561**:811-819

[102] Holowatz LA, Thompson CS, Minson CT, Kenney WL. Mechanisms of acetylcholine-mediated vasodilatation in young and aged human skin. The Journal of Physiology. 2005;**563**:965-973

[103] Noon JP, Walker BR, Hand MF, Webb DJ. Studies with iontophoretic administration of drugs to human dermal vessels in vivo: Cholinergic vasodilatation is mediated by dilator prostanoids rather than nitric oxide. British Journal of Clinical Pharmacology. 1998;**45**:545-550

[104] Berghoff M, Kathpal M, Kilo S, Hilz MJ, Freeman R. Vascular and neural mechanisms of ACh-mediated vasodilation in the forearm cutaneous microcirculation. Journal of Applied Physiology. 2002;**92**:780-788

[105] Durand S, Fromy B, Bouye P, Saumet JL, Abraham P. Current-induced vasodilation during water iontophoresis (5 min, 0.10 mA) is delayed from current onset and involves aspirin sensitive mechanisms. Journal of Vascular Research. 2002;**39**:59-71

[106] Ferrell WR, Ramsay JE, Brooks N, Lockhart JC, Dickson S, McNeece GM, Greer IA, Sattar N. Elimination of electrically induced iontophoretic artefacts: Implications for non-invasive assessment of peripheral microvascular function. Journal of Vascular Research. 2002;**39**:447-455

[107] Agarwal SC, Allen J, Murray A, Purcell IF. Comparative reproducibility of dermal microvascular blood flow changes in response to acetylcholine iontophoresis, hyperthermia and reactive hyperaemia. Physiological Measurement. 2010;**31**:1-11

[108] Blaise S, Hellmann M, Roustit M, Isnard S, Cracowski JL. Oral sildenafil increases skin hyperaemia induced by iontophoresis of sodium nitroprusside in healthy volunteers. British Journal of Pharmacology. 2010;**160**:1128-1134

[109] Roustit M, Blaise S, Cracowski JL. Sodium nitroprusside iontophoresis on the finger pad does not consistently increase skin blood flow in healthy controls and patients with systemic sclerosis. Microvascular Research. 2009;**77**:260-264

[110] Larkin SW, Williams TJ. Evidence for sensory nerve involvement in cutaneous reactive hyperemia in humans. Circulation Research. 1993;**73**:147-154

[111] Wong BJ, Wilkins BW, Holowatz LA, Minson CT. Nitric oxide synthase inhibition does not alter the reactive hyperemic response in the cutaneous circulation. Journal of Applied Physiology. 2003;**95**:504-510

[112] Yvonne-Tee GB, Rasool AH, Halim AS, Rahman AR. Reproducibility of different laser Doppler fluximetry parameters of postocclusive reactive hyperemia in human forearm skin. Journal of Pharmacological and Toxicological Methods. 2005;**52**:286-292

[113] Yvonne-Tee GB, Rasool AH, Halim AS, Wong AR, Rahman AR. Method optimization on the use of postocclusive hyperemia model to assess microvascular function. Clinical Hemorheology and Microcirculation. 2008;**38**:119-133

[114] Keymel S, Sichwardt J, Balzer J, Stegemann E, Rassaf T, Kleinbongard P, Kelm M, Heiss C, Lauer T. Characterization of the non-invasive assessment of the cutaneous microcirculation by laser Doppler perfusion scanner. Microcirculation. 2010;**17**:358-366

[115] Minson CT, Berry LT, Joyner MJ. Nitric oxide and neurally mediated regulation of skin blood flow during local heating. Journal of Applied Physiology. 2001;**91**:1619-1626

[116] Johnson JM, Kellogg DL Jr. Local thermal control of the human cutaneous circulation. Journal of Applied Physiology 2010;**109**:1229-1238

[117] Briers JD. Laser Doppler, speckle and related techniques for blood perfusion mapping and imaging. Physiological Measurement. 2001;**22**:R35-R66

[118] Roustit M, Millet C, Blaise S, Dufournet B, Cracowski JL. Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity. Microvascular Research. 2010;**80**:505-511

[119] Ahmadi N, McQuilkin GL, Akhtar MW, Hajsadeghi F, Kleis SJ, Hecht H, Naghavi M, Budoff M. Reproducibility and variability of digital thermal monitoring of vascular reactivity. Clinical Physiology and Functional Imaging. 2011;**31**(6):422-428

[120] Gul KM, Ahmadi N, Wang Z, Jamieson C, Nasir K, Metcalfe R. Digital thermal monitoring of vascular function: A novel tool to improve cardiovascular risk assessment. Vascular Medicine. 2009;**14**(2):143-148

[121] Ahmadi N, Hajsadeghi F, Gul K, Vane J, Usman N, Flores F, Nasir K, Hecht H, Naghavi M, Budoff M. Relations between digital thermal monitoring of vascular function, the Framingham risk score, and coronary artery calcium score. Journal of Cardiovascular Computed Tomography. 2008;**2**(6):382-388

[122] Alley H, Owens CD, Gasper WJ, Grenon SM. Ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery in clinical research. Journal of Visualized Experiments . 2014, 22;(92):e52070

[123] Parker BA, Tschakovsky ME, Augeri AL, Polk DM, Thompson PD, Kiernan FJ. Heterogenous vasodilator pathways underlie flow-mediated dilation in men and women. The American Journal of Physiology. 2011;**301**:H1118-H1126

[124] Bretón-Romero R, Wang N, Palmisano J, Larson MG, Vasan RS, Mitchell GF, Benjamin EJ, Vita JA, Hamburg NM. Cross-sectional associations of flow reversal, vascular function, and arterial stiffness in the Framingham heart study. Arteriosclerosis, Thrombosis, and Vascular Biology. 2016;**36**(12):2452-2459

[125] Takase B, Uehata A, Akima T, Nagai T, Nishioka T, Hamabe A, Satomura K, Ohsuzu F, Kurita A. Endothelium-dependent flow-mediated vasodilation in coronary and brachial arteries in suspected coronary artery disease. Am J Cardiol. 1998, 15;**82**(12):1535-9,A7-8

[126] Sorensen KE, Celermajer DS, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Thomas O, Deanfield JE. Non-invasive measurement of human endothelium dependent arterial responses: Accuracy and reproducibility. British Heart Journal. 1995;**74**(3):247-253

[127] Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: A report of the international brachial artery reactivity task force. Journal of the American College of Cardiology. 2002;**39**:257-265

[128] Deanfield J, Donald A, Ferri C, Giannattasio C, Halcox J, Halligan S, Lerman A, Mancia G, Oliver JJ, Pessina AC, Rizzoni D, Rossi GP, Salvetti A, Schiffrin EL, Taddei S, Webb DJ. Endothelial function and dysfunction, part I: Methodological issues for assessment in the different vascular beds: A statement by the Working Group on Endothelin and Endothelial Factors of the European Society of Hypertension. Journal of Hypertension. 2005;**23**:7-17

[129] Maruhashi T, Soga J, Fujimura N, et al. Nitroglycerine-induced vasodilation for assessment of vascular function: A comparison with flow-mediated vasodilation. Arteriosclerosis, Thrombosis, and Vascular Biology. 2013;**33**:1401-1408

[130] Inoue T, Matsuoka H, Higashi Y, et al. Flow-mediated vasodilation as a diagnostic modality for vascular failure. Hypertension Research. 2008;**31**:2105-2113

[131] Raitakari OT, Seale JP, Celermajer DS. Impaired vascular responses to nitroglycerin in subjects with coronary atherosclerosis. The American Journal of Cardiology. 2001;**87**(2):217-219

[132] Schächinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. Circulation. 2000;**101**:1899-1906

[133] Ukawa T, Takayanagi T, Morimoto H, Higashi Y, Idei N, Yoshizumi M, Tsuji T. Novel non-invasive method of measurement of endothelial function: Enclosed-zone flow-mediated dilatation (ezFMD). Medical & Biological Engineering & Computing. 2012;**50**(12):1239-1247

[134] Idei N, Ukawa T, Hata T, et al. A novel noninvasive and simple method for assessment of endothelial function: Enclosed zone flow-mediated vasodilation (ezFMD) using an oscillation amplitude measurement. Atherosclerosis. 2013;**229**:324-330

[135] Tschudi M, Richard V, Buhler FR, Luscher TF. Importance of endothelium-derived nitric oxide in porcine coronary resistance arteries. The American Journal of Physiology. 1991;**260**:H13-H20

[136] Nabel EG, Ganz P, Gordon JB, Alexander RW, Selwyn AP. Dilation of normal and constriction of atherosclerotic coronary arteries caused by the cold pressor test. Circulation. 1988;**77**:43-52

[137] Laurent S, Cockcroft J, Van BL, et al. Expert consensus document on arterial stiffness: Methodological issues and clinical applications. European Heart Journal. 2006;**27**(21):2588-2605

[138] Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: A systematic review and meta-analysis. Journal of the American College of Cardiology. 2010;**55**(13):1318-1327

[139] Ben-Shlomo Y, Spears M, Boustred C, May M, Anderson SG, Benjamin EJ, Boutouyrie P, Cameron J, Chen CH, Cruickshank JK, Hwang SJ, Lakatta EG, Laurent S, Maldonado J, Mitchell GF, Najjar SS, Newman AB, Ohishi M, Pannier B, Pereira T, Vasan RS,

Shokawa T, Sutton-Tyrell K, Verbeke F, Wang K, Webb DJ, Willum Hansen T, Zoungas S, McEniery CM, Cockcroft JR, Wilkinson IB. Aortic pulse wave velocity improves cardiovascular event prediction: An individual participant meta-analysis of prospective observational data from 17,635 subjects. Journal of the American College of Cardiology. 2014;**63**(7):636-646

[140] Vlachopoulos C, O'Rourke M. Genesis of the normal and abnormal arterial pulse. Current Problems in Cardiology. 2000;**25**(5):303-367

[141] Husmann M, Jacomella V, Thalhammer C, Amann-Vesti BR. Markers of arterial stiffness in peripheral arterial disease. VASA. 2015;**44**:341-348

[142] Stein JH, Korcarz CE, Hurst RT, Lonn E, Kendall CB, Mohler ER, Najjar SS, Rembold CM, Post WS. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: A consensus statement from the American Society of Echocardiography carotid intima-media thickness task force. Endorsed by the Society for Vascular Medicine. Journal of the American Society of Echocardiography. 2008;**21**(2):93-111

[143] Chambless LE, Heiss G, Folsom AR, Rosamond W, Szklo M, Sharrett AR, Clegg LX. Association of coronary heart disease incidence with carotid arterial wall thickness and major risk factors: The atherosclerosis risk in communities (ARIC) study, 1987-1993. American Journal of Epidemiology. 1997;**146**(6):483-494

[144] O'Leary DH, Polak JF, Kronmal RA, Manolio TA, Burke GL, Wolfson SK Jr. Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. Cardiovascular health study collaborative research group. The New England Journal of Medicine. 1999;**340**(1):14-22

[145] Finn AV, Kolodgie FD, Virmani R. Correlation between carotid intimal/ medial thickness and atherosclerosis: A point of view from pathology. Arteriosclerosis, Thrombosis, and Vascular Biology. 2010;**30**(2):177-181

[146] Onut R, Balanescu AP, Constantinescu D, Calmac L, Marinescu M, Dorobantu PM. Imaging atherosclerosis by carotid intima-media thickness in vivo: How to, where and in whom? Maedica (Buchar). 2012;**7**(2):153-162

[147] Mudau M, Genis A, Lochner A, Strijdom H. Endothelial dysfunction: The early predictor of atherosclerosis. CVJ Africa. 2012;**23**(4):222-229

[148] Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: Testing and clinical relevance. Circulation. 2007;**115**:1285-1295