

## SEMINAR FOR PHISICIANS *SEMINAR ZA LEKARE U PRAKSI*

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Seminar za lekare u praksi  
*Seminar for phisicians*  
UDK 616.1: 577.2 i 616.1-092  
DOI: 10.2298/MPNS1702053S

### BIOMARKERS OF ENDOTHELIAL DYSFUNCTION IN CARDIOVASCULAR DISEASES

#### *BIOMARKERI ENDOTELNE DISFUNKCIJE U BOLESTIMA KARDIOVASKULARNOG SISTEMA*

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#### Summary

**Introduction.** Endothelial dysfunction is the result of numerous infectious or noninfectious acute and chronic diseases, mechanical damage, hemodynamic imbalance and effects of certain drugs. Endothelial dysfunction can be assessed by determining biomarkers (adhesion molecules, inflammatory cytokines and growth factors, and noninvasive visualization biomarkers). **Intercellular adhesion molecule -1 and vascular cellular adhesion molecule.** Adhesion molecules mediate the interaction of cells with the extracellular matrix, as well as with other cells. It is shown that adhesion molecules and molecules of the extracellular matrix are markers of endothelial dysfunction and they are involved in the pathogenesis of atherosclerosis. **P-selectin and E-selectin.** Determination of these two mediators is important not only in the evaluation of endothelial damage in acute inflammation, but in other chronic non-infectious conditions such as atherosclerosis. **C-reactive protein.** C-reactive protein reduces the transcription of endothelial nitric oxide synthase at the level of the endothelial cells and 'destabilize' their messenger ribonucleic acid, thus leading to a reduction of the synthesis of nitric oxide, in the basal and stimulated conditions, which is significant for the development of endothelial dysfunction as a part of the formation of subclinical atherosclerosis. **Vascular endothelial growth factor, fibrinogen and thrombomodulin.** Vascular endothelial growth factor is a cytokine that stimulates angiogenesis for the purpose of revascularization of ischemic tissues, and mediates in a variety of functions of endothelial cells, including proliferation, migration, invasion, survival and permeability. Noninvasive visualization biomarkers. Determination of intima-media complex thickness and calcium score index by computed tomography are considered clinically reliable methods in prevention and early diagnosis of coronary artery disease and acute myocardial infarction, changing the basic concepts of prevention. **Conclusion.** At this point it is not easy to say what clinical significance are listed biomarkers of endothelial dysfunction in determining the risk for cardiovascular disease.

**Key words:** Biomarkers; Endothelium, Vascular; Risk Factors; Cardiovascular Diseases; C-Reactive Protein; Selectins; Intercellular Adhesion Molecule-1; Vascular Cell Adhesion Molecule-1; Vascular Endothelial Growth Factor A; Thrombomodulin

#### Sažetak

**Uvod.** Endotelna disfunkcija je posledica brojnih infektivnih ili neinfektivnih akutnih ili hroničnih bolesti, mehaničkog oštećenja, hemodinamskog disbalansa i delovanja nekih lekova. Endotelna disfunkcija se može procenti određivanjem biomarkera endotelne disfunkcije (adhezijskih molekula, selektina, faktora rasta i zapaljenja, neinvazivnim vizuelizacionim biomarkerima). **Intercelularni adhezioni molekul-1 i vaskularni adhezioni molekul.** Adhezioni molekuli posreduju u interakciji ćelija sa ekstracelularnim matriksom, kao i sa drugim ćelijama. I za adhezijske molekule i za molekule ekstracelularnog matriksa pokazano je da su markeri endotelne disfunkcije te da su uključeni u patogenezu ateroskleroze. **P-selectin and E-selectin.** Određivanje ova dva biomarkera značajno je u proceni oštećenja endotela u akutnom zapaljenju, ali i u drugim hroničnim neinfektivnim stanjima, poput ateroskleroze. **C/ reaktivni protein.** C-reaktivni protein smanjuje transkripciju eNOS-a na nivou endotelnih ćelija i "destabilize" njihovu mRNK, dovodeći na taj način do smanjenja sinteze azot-monoksida, u bazalnim i stimulisanim uslovima. **Vaskularni endotelni faktor rasta, fibrinogen i trombomodulin.** Vaskularni endotelni faktor rasta je citokin koji stimuliše angiogenezu u svrhu revaskularizacije ishemičnih tkiva, a posreduje u brojnim funkcijama endotelnih ćelija, uključujući proliferaciju, migraciju, invaziju, preživljavanje i permeabilnost. Neinvazivni vizuelizacioni biomarkeri. Klinički pouzdani u prevenciji i ranoj dijagnozi bolesti koronarnih arterija i akutnog infarkta miokarda jeste određivanje intimomedijalnog kompleksa i određivanje Ca Scor indeksa pregledom kompjuterizovanom tomografijom, koji su izmenili bazične koncepte mogućnosti prevencije. **Zaključak.** U ovom trenutku rano je reći kakvu kliničku vrednost imaju pojedini biomarkeri endotelne disfunkcije u određivanju rizika za oboljevanje od kardiovaskularnih bolesti.

**Ključne reči:** biomarkeri; vaskularni endotel; faktori rizika; kardiovaskularne bolesti; CRP; selektin; intercelularni adhezijski molekul-1; vaskularno celularni adhezijski molekul-1; vaskularni endotelni faktor rasta; trombomodulin

### Abbreviations

ED	– endothelial dysfunction
ICAM-1	– intercellular adhesion molecule-1
VCAM-1	– vascular cellular adhesion molecule-1
VEGF	– vascular endothelial growth factor
CRP	– C-reactive protein
LFA	– lymphocyte function associated antigen 1
NO	– nitric oxide
IL	– interleukin
IMP	– intima media complex

### Introduction

Endothelial dysfunction (ED) is an early, usually asymptomatic phase of endothelial cell damage which results in disruption of its numerous functions. During this process, various toxic substances induce the loss of protective mechanisms of the endothelium, including: anti-platelet and anti-inflammatory functions. It affects proliferation, migration and survival of endothelial cells, and endothelial permeability change [1]. ED effects are first manifested at the microvascular level of different tissues in any organ system, in extremely large number of infectious and non-infectious, acute or chronic diseases. The vascular endothelium is a single-cell layer forming the inner lining of the blood vessel which has a protective function but it can be compromised by activation of cytokines and other inflammatory mediators. ED is also caused by other potentially damaging factors: mechanical damage, hemodynamic imbalance and certain medications [2]. It is well known that aging and diabetes mellitus are two independent risk factors for the development of ED, while the concept of low-intensity chronic inflammation includes other conditions that can lead to ED, as well as infectious or non-infectious diseases, hypertension, dyslipidemia, smoking, etc. [3]. Each of these conditions may be initiated by the expression of adhesion molecules, enhanced release of cytokines, chemokines, growth factors and other inflammatory mediators that promote migration and activation of inflammatory cells.

Important biomarkers and mediators of ED are endothelial adhesion molecules, growth factors, and inflammatory factors. They participate not only in local, but in systemic response to tissue damage, as well. The ED biomarkers include: intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), P- and E-selectin, vascular endothelial growth factor (VEGF) and C-reactive protein (CRP) [4]. Recent studies indicate a significant contribution of ED in the pathogenesis of atherosclerosis and cardiovascular disease (CVD). Atherosclerotic plaque formation includes a greater number of cells: platelets, endothelial cells, activated monocytes, macrophages and smooth muscle cells. Macrophages and T-lymphocytes play a key role in the growth and changes of atherosclerotic plaque, mediated by the secretion of growth factors that stimulate the proliferation of smooth muscle cells and secretion of the extracellular matrix, proinflammatory cytokines, interferon- $\gamma$  or interleukin-1, with a si-

multaneous activation of enzymes that break down the extracellular matrix, such as metalloproteinases, causing weakening of the fibrous part of the plaque [5].

Numerous studies have found a positive correlation between ED and coronary artery disease. It is believed that ED, alone, without coronary disease and without evidence of coronary artery spasm, is caused by the acute coronary syndrome, unstable angina and myocardial infarction, without ST segment elevation. One of the potentially most important factors in the development of ED is insufficient production of nitric oxide (NO), vasodilator of endothelial origin [6]. The term “microvascular coronary dysfunction” has become a distinct clinical entity, which refers to ischemia with clinical signs of angina, defined by the triad of chest pain, evidence of ischemia by stress testing, and non-obstructive coronary artery disease.

Currently there is no gold standard for the assessment of ED. Ultrasound measured flow-mediated dilatation (FMD) of the brachial artery has proven to be a useful non-invasive method. Venous occlusion plethysmography, determination of the intima-media complex thickness, pulse wave velocity and peritoneal equilibrium test (PET) are some of the methods, but rarely used in practice. The future of the assessment of ED is in determining the level of biomarkers, adhesion molecules (VCAM, ICAM-1, selectins), and VEGF, fibrinogen, thrombomodulin and CRP in the blood.

### Intercellular adhesion molecule-1 and vascular cellular adhesion molecule-1

Vascular cellular adhesion molecule-1 and ICAM-1, (also known as CD54) are adhesion molecules of similar structure and function. VCAM-1 gene contains seven immunoglobulin domains and it is expressed on all blood vessels, regardless of their size. However, upon stimulation of endothelial cells by cytokines its concentration is significantly increased. In addition to the endothelial cells, VCAM-1 is expressed in neurons, smooth muscle cells, fibroblasts and macrophages [4].

Synthesis and secretion of ICAM-1 can be induced by interleukin-1 (IL-1) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and it functions as a ligand for lymphocyte function associated antigen 1 (LFA-1) (integrin) receptor found on leukocytes. Upon activation, leukocytes bind to endothelial cells via the ICAM-1/LFA-1, and this allows them to migrate into the tissues. Elevated levels of ICAM-1 were observed in patients with type II diabetes mellitus, cardiovascular diseases, graft dysfunction, oxidative stress, abdominal obesity, hypertension, liver disease and some malignant diseases. Intercellular adhesion molecule-1 promotes angiogenesis and studies have shown that it can be an indicator of activation or damage of endothelial cells [7].

Adhesion molecules mediate the interaction of cells with the extracellular matrix, as well as with other cells. It is shown that both adhesion molecules and extracellular matrix molecules are markers of ED and are involved in the pathogenesis of atherosclerosis. Also, immunohistochemical analysis of

atherosclerotic plaque demonstrated the presence of a number of adhesion molecules [5]. At the site of inflammation, proinflammatory cytokines lead to increased expression of ICAM-1 on vascular endothelium and activation of leukocyte integrins, which results in the adhesion of leukocytes to endothelial cells and migration to the site of inflammation. VCAM-1 expression is found in small blood vessels after stimulation of endothelial cells with cytokines, and patients with coronary heart disease showed higher levels of VCAM-1 than those with normal epicardial coronary arteries [8]. In the previous studies of ED and coronary artery disease biomarkers, the best tested was ICAM-1. People with higher basal levels of ICAM-1 had a two-fold risk of developing cardiovascular disease and in the absence of other factors for cardiovascular disease, which marked this biomarker of ED as a potential indicator of future ischemic incident; further researches could confirm its clinical value in determining cardiovascular risk and mortality. An increase in the value of ICAM-1 has been established within the first 12 - 24 hours after acute myocardial infarction and during ischemia and reperfusion [8]. Studies have also shown a connection between higher values of ICAM-1 and the occurrence of reperfusion arrhythmias. The serum concentration of this biomarker is reduced in individuals using medications, such as angiotensin converting enzyme inhibitors (ACE inhibitors) and calcium channel blockers. VCAM-1 may be independently elevated in asymptomatic smokers as well, while the elevated value of this adhesion and E-selectin were observed in both active and former asymptomatic smokers [10].

It has been determined that patients with elevated plasma VCAM-1 concentration and stable coronary heart disease are at higher risk of future cardiovascular events. In patients hospitalized for acute coronary syndrome, higher values of this marker are correlated with intrahospital complications, including a fatal myocardial infarction [5, 8, 9]. Both of these markers of plasma endothelial dysfunction are closely associated with total mortality from cardiovascular disease.

There are several factors that lead to elevation in plasma concentrations of adhesion molecules: hypertension, immunosuppressive therapy, autoimmune disease and graft rejection [11]. In experimental conditions, both types of adhesion molecules are expressed on the endothelium of the aorta in the regions predisposed to atherosclerosis. They can be easily measured in plasma by enzyme-linked immunosorbent assay (ELISA test) and thus represent potential biomarkers indicating endothelial activation and vascular inflammation.

### **P-selectin and E-selectin**

P-selectin and E-selectin play a major role in the migration and chemotaxis of lymphocytes in the tissues and neutrophils in the formation of thrombus in atherosclerosis. Determination of these two mediators is important not only in the evaluation of endothelial damage in acute infectious inflamma-

tion, but in other chronic non-infectious conditions such as atherosclerosis, as well. They were found in Weibel-Palade bodies of the endothelial cells and alpha-granules of platelets. These molecules are important in the activation of T- and B-lymphocytes and adhesion of platelets, monocytes and neutrophils, which play a central role in the accumulation of neutrophils within the thrombus [10].

E-selectin expression is found only on endothelial cells after activation of proinflammatory cytokines (interleukin-1, tumor necrosis factor) or endotoxin. Although elevated values of P-selectin are indicators of future coronary events in women, and elevated basal levels of ED mediators are observed in men, studies have not shown a sufficient degree of predictive value for their clinical use. It is believed that E-selectin is an important biomarker for monitoring ischemia and necrosis after acute myocardial infarction. In case of acute ischemic event, high levels of ICAM-1, VCAM-1, P- and E-selectin may be found, all of which remain elevated over the next six months, followed by a return to the baseline. In case of myocardial revascularization, the value of ICAM-1 and P-selectin remain elevated for about 24 months after the surgical procedure [12].

### **C-reactive protein**

C-reactive protein is a biomarker of inflammation and ED. A CRP test helps in determining ED, which separates it from other serum indicators of ED. CRP is one of the leading acute phase proteins of inflammation and a marker of systemic inflammatory response. It is elevated in certain chronic diseases, including atherosclerosis, and it is believed that this protein, synthesized in the liver, causes and prolongs the inflammatory component of atherosclerosis by activation of a number of molecules present in/on endothelial cells which participate in the process of atherogenesis [13].

The physiological role of this protein is to identify potentially toxic autogenous substances released from damaged tissues, to bind them and then detoxify them or remove from the blood. A considerable number of studies have indicated that high-sensitivity CRP (hsCRP) is one of the most significant independent predictors for the occurrence of adverse vascular events [8, 14]. CRP reduces eNOS transcription at the level of the endothelial cells, and destabilizes eNOS messenger ribonucleic acid (mRNA), thus leading to a reduction of NO synthesis, in the basal and stimulated conditions, which is significant for the development of ED as part of the subclinical atherosclerosis [6]. This molecule also stimulates the release of endothelin (ET-1) and IL-6, elevates the expression of adhesion molecules, stimulates monocyte chemoattractant protein-1 and facilitates the entry of LDL into macrophages, thus participating in their transformation into foam cells, which is an important stage in the atherogenesis [14]. Researches show that CRP facilitates apoptosis of endothelial cells, which is described as part of the mechanism of ED. In addition to direct effects on the promotion of endothelial acti-

vation, CRP also indirectly inhibits bone-marrow derived endothelial progenitor cells and thus prevents repair of the endothelium. Besides the formation of plaque, CRP has a role in its further maintenance, leading to elevated expression of receptors for angiotensin and on vascular smooth muscle cells (*in vivo* and *in vitro*), stimulating their proliferation, neointimal formation and reactive oxygen species.

Statins exhibit positive effects on elevated levels of CRP in patients with statin-treated hyperlipidemia, which makes them useful substances in the primary prevention of coronary heart disease in patients with slightly elevated or normal lipidogram values [15]. Many studies have determined a correlation between elevated CRP levels and progression of atherosclerosis. In particular, a positive correlation was observed between the above mentioned, the severity of coronary artery disease and the risk for development of acute coronary syndrome. However, researchers disagree over the connection between cardiovascular diseases and CRP. CRP is an indicator of ED, but it is not clear whether it is a predictor of cardiovascular events, given the heterogeneity of causes and mechanisms of ED occurrence.

### **Vascular endothelial growth factor, fibrinogen and thrombomodulin**

Vascular endothelial growth factor (VEGF) is a cytokine which promotes angiogenesis and ischemic tissue revascularization, and mediates in a variety of functions of endothelial cells, including proliferation, migration, invasion, survival and permeability. During the intracoronary thrombus formation, platelets release significant amounts of VEGF, and there is a significant positive correlation between the serum values of VEGF and platelet count and the size of thrombus in patients with STEMI myocardial infarction. Higher values of this factor were recorded in acute coronary events in relation to chronic ischemic conditions, and it is believed that the reason for this is under-developed collateral circulation in patients who develop an acute incident. It was determined that heparin simultaneously binds VEGF and endothelial cells, thus suppressing ED and lowering serum concentration of VEGF [16, 17].

A high level of fibrinogen is associated with an increased risk for developing cardiovascular disease in both men and women, but some differences were detected in relation to gender. The level of plasma fibrinogen is elevated in menopause, during the use of oral contraceptives, and pregnancy, although the hormone replacement therapy affects its reduction [18]. The latest European guidelines on cardiovascular disease prevention in clinical practice recommend determination of fibrinogen as a part of risk assessment in patients with low or moderate risk for developing cardiovascular disease [18].

Thrombomodulin (TM) is traditionally thought to be a biomarker of ED. It is a transmembrane glycoprotein which is expressed on vascular endothelial cells and represents a thrombin receptor. A high level of thrombomodulin is associated with elevated

level of plasminogen activator inhibitor (plasminogen activator inhibitor-1, PAI-1) [20, 21]. Thrombomodulin and von Willebrand factor are transferred to plasma and their high values are found in patients with endothelial dysfunction suffering from type II diabetes mellitus.

### **Noninvasive visualization**

Noninvasive imaging biomarkers are a valuable tool in the diagnosis of cardiovascular diseases. The determination of intima-media complex thickness (IMC) and Ca Score Index by computed tomography examination are considered clinically reliable methods in prevention and early diagnosis of coronary artery disease and acute myocardial infarction, affecting the change in the basic concepts of prevention.

An integral part of the Doppler ultrasonography of blood vessels is determining intima-media complex thickness. IMC is measured in common, internal carotid arteries, or on the walls of the carotid bulb. In clinical practice the significance of this parameter is well defined, and since normal IMC values vary significantly according to gender, age and ethnicity, they are defined for different geographic areas [22].

Calcium accumulation in the walls of blood vessels is an active cellular process and an integral part of atherosclerosis. In physiological conditions, the wall of the coronary artery does not contain calcium. The presence of calcium in the coronary arteries is a sign of coronary artery disease. There is a direct linear relationship between the calcium concentration and the degree of atherosclerosis, which is determined by Ca Score Index. Detection of calcium in the walls of coronary arteries is a certain sign of some degree of atherosclerosis [23].

### **Conclusion**

Endothelial dysfunction, manifesting as a subclinical atherosclerosis, occurs long before structural changes to the vessel wall are apparent, and represents an independent risk factor for future occurrence of cardiovascular events. The tissue damage is a result of the accumulation of inflammatory cells. Also, an activation mechanism leads to the expression of different adhesion molecules and chemokines as signalling molecules involved in the recruitment of inflammatory cells in the target tissues. If such changes occur in the myocardium, it leads to the development of fibrosis, which manifests by diastolic dysfunction. Endothelial cells play a leading role in the process of inflammation, because they represent a direct barrier between the target tissue and circulating inflammatory cells. If the cell is stimulated by the pro-inflammatory signals: cytokines, endotoxin, modified lipids or reactive oxygen species, it causes activation of an inhibitor necrosis factor of kappa B kinases and the expression of pro-inflammatory mediators: intercellular adhesion molecule-1, vascular cellular adhesion molecule-1, monocyte chemoattractant protein-1, interleukin-6 and others. At the same time, it

inhibits nitric oxide production. These endothelial changes lead to chronic inflammation in the walls of blood vessels and play a key role in the development of atherosclerosis. Future researches should be directed towards the clarification of the possible role of a signaling molecules mediators of endothelial dysfunction, as potential predictors of future cardiovascular events, both in persons diagnosed with cardiovascular disease,

and in those who still have asymptomatic atherosclerosis. The mechanisms of action of certain endothelial dysfunction markers are still not fully elucidated and require further research to understand their role and the mode of operation in all respects. At this point, it is too soon to evaluate the clinical significance of mediators and biomarkers of endothelial dysfunction in determining the risk for cardiovascular disease.

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Rad je primljen 23. V 2016.

Recenziran 21. VIII 2016.

Prihvaćen za štampu 2. XI 2016.

BIBLID.0025-8105:(2017):LXX:1-2:53-57.