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# INFLAMMATORY AND APOPTOTIC MARKERS IN ISCHEMIC HEART DISEASE PATIENTS

## MARKERI INFLAMACIJE I APOPTOZE KOD BOLESNIKA SA ISHEMIJSKOM BOLEŠĆU SRCA

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**Summary:** Ischemic heart disease is the most frequent cause of cardiovascular morbidity and mortality. It is developed on the basis of atherosclerosis which is today considered a chronic inflammatory disease. It is documented by an increase in inflammatory and immune biomarkers, such as C-reactive protein, fibrinogen, neopterin, leukocytes, lymphocytes and others, that are significantly changed in patients with unstable angina or acute myocardial infarction. CRP is mostly studied. Increased concentrations of CRP are associated with a series of risk factors. CRP may predict recurrent events and mortality independently of cardiac troponin levels, and it is also an independent predictor of a cardiovascular event after adjustment for traditional risk factors. Although CRP currently appears to be the most promising biological marker, there is still controversy regarding its use in clinical practice. Both necrotic and apoptotic cell death are documented during atherogenesis, however, limited data are available about apoptotic markers in ischemic heart disease patients. Increasing evidence supports the existence of apoptotic death initiated by ligation of membrane-bound death receptors or by release of cytochrome c from mitochondria, as well as their regulators in the heart. The studies of serum markers show that the apoptotic process is disregulated in ischemic heart disease patients. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is present in stable atherosclerotic lesions, is increased in vulnerable plaques, but its serum levels are reduced significantly in patients with unstable angina. Serum Fas concentrations are increased and FasL are decreased in subjects at high cardiovascular risk. The results of our study show significant changes in serum Fas, FasL, and Bcl-2 concentrations, and lymphocyte caspase-3 activity in different stages of ischemic heart disease. For now, there is evidence that statins are effective in the regulation of some apoptotic markers. The better understanding of the pathways of apoptosis and their regulation is promissing in yielding novel therapeutic targets for cardiovascular disease.

**Keywords:** inflammatory markers, apoptotic markers, ischemic heart disease

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**Kratak sadržaj:** Ishemijska bolest srca je najčešći uzrok kardiovaskularnog morbiditeta i mortaliteta. Razvija se na terenu ateroskleroze koja se danas smatra hroničnom inflamatornom bolešću. Inflamacija je dokazana praćenjem inflamatornih i imunih biomarkera kao što su C-reaktivni protein (CRP), fibrinogen, neopterin, leukociti, limfociti i drugi koji se značajno menjaju kod bolesnika sa nestabilnom anginom pektoris ili akutnim infarktom miokarda. Najčešće ispitivan marker je CRP. Povećane koncentracije CRP su udružene sa brojnim faktorima rizika. Na osnovu vrednosti CRP mogu se predvideti rekurentni događaji i mortalitet nezavisno od vrednosti srčanog troponina, a takođe je nezavisan prediktor kardiovaskularnog događaja nakon korekcije tradicionalnih faktora rizika. Iako se CRP trenutno smatra biološkim markerom koji najviše obećava, još uvek postoje nedoumice u vezi sa njegovim korišćenjem u kliničkoj praksi. Mada je dokazano da se i nekroza i apoptoza dešavaju u toku aterogeneze, malo je dostupnih podataka koji se odnose na markere apoptoze kod bolesnika sa ishemijskom bolešću srca. Sve je više informacija koje ukazuju da se u srcu odvijaju apoptoza kao i njena regulacija, i da se apoptoza može inicirati preko membranskih receptora smrti, ali i oslobađanjem citohroma c iz mitohondrija. Ispitivanje serumskih markera je pokazalo da je proces apoptoze poremećen kod bolesnika sa ishemijskom bolešću srca. TRAIL (*tumor necrosis factor-related apoptosis-inducing ligand*) prisutan je u stabilnim aterosklerotskim lezijama, povećan u vulnerabilnim plakovima, dok su njegove serumske vrednosti značajno snižene kod bolesnika sa nestabilnom anginom. Koncentracija serumskog Fas je povećana, a FasL snižena kod osoba sa visokim rizikom. Rezultati naše studije su pokazali značajne promene koncentracija Fas, FasL i Bcl-2 u serumu, kao i aktivnost kaspaze-3 u limfocitima u različitim stadijumima ishemijske bolesti srca. Za sada postoje podaci o efektivnosti statina u regulaciji nekih markera apoptoze. Bolje razumevanje puteva apoptoze i njihove regulacije može omogućiti nov terapijski pristup kardiovaskularnim bolestima.

**Ključne reči:** markeri inflamacije, markeri apoptoze, ishemijska bolest srca

## Introduction

Despite the changes in lifestyle and the use of new pharmacological drugs to lower plasma lipid concentration (1, 2), cardiovascular diseases continue to be the principal cause of death in the United States, Europe, and much of Asia (3, 4). Among cardiovascular diseases, ischemic heart disease occurs most frequently. Atherosclerosis is validated in more than 90% of these patients. It is well known that inflammation plays a role in the initiation and the evolution of atherogenesis (5). Whatever the initial stimuli (mechanical, chemical, infectious or immunological), which depends on the nature of risk factors, a continuous ongoing inflammatory process leads to the evolution of an uncomplicated atheromatous plaque into complex and vulnerable atheroma. A flared plaque inflammation is considered a source of intimal erosion and rupture and therefore of acute ischemia (6). These studies are supported by the clinical findings of increased inflammatory markers in patients with chronic stable angina (7), unstable angina pectoris (8), and acute myocardial infarction (6). Furthermore, acute coronary syndrome is associated with both necrotic and apoptotic cell death. Necrosis is followed by a significant increase in troponines whilst apoptotic markers have not been well established yet. Also, there is evidence of the predictive value of inflammatory markers for subsequent coronary events (6).

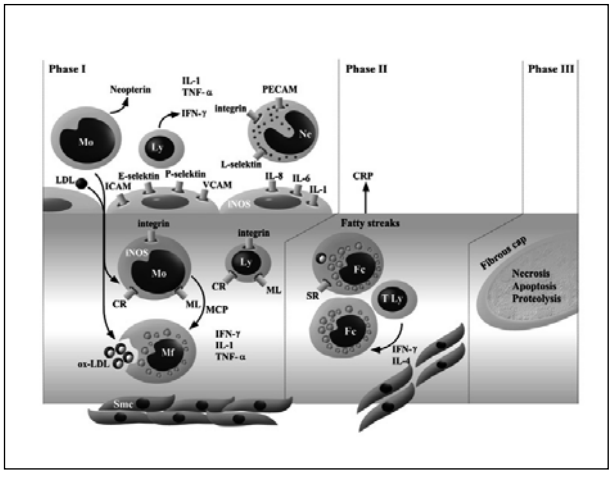


**Figure 1** Inflammatory molecules production during evolution of atherosclerosis (I – endothelial dysfunction; II – fatty streak formation; III – formation of an advanced atherosclerotic lesion).

## Inflammatory markers

Inflammatory, immune and native vascular wall cells are implicated in all stages of atherogenesis via the production of cytokines, chemoattractants, adhesive molecules and other biomolecules such as C-reactive protein (CRP), neopterin, nitric oxide (NO), cystatin C, and the pregnancy associated plasma protein A (PAPP-A) (9). Monocytes and lymphocytes are the first cells involved in the initiation of atherogenesis, which produce inflammatory cytokines leading to the endothelial activation or dysfunction. Hallmarks of the chronic atherosclerotic process are also monocyte-macrophage and lymphocyte cells, initially found in the fatty streaks. They also play a major role in plaque rupture and superimposed thrombosis (5, 10, 11). Generally, biomarkers of atherosclerosis include: inflammatory biomarkers, indicators of immune system activation, cytokines, and markers of autoimmunity (*Table I*). Markers and inflammatory mediators are related to acute phase reactants (APR) such as CRP, fibrinogen, serum amyloid A (SAA), albumin and leukocyte count.

**Table I** Biomarkers and mediators of inflammation and atherosclerosis.

| | |
|---|---|
| I Acute-phase reactants: | – CRP (C-reactive protein)<br>– Fibrinogen<br>– SAA (Serum amyloid A)<br>– Albumin<br>– Leukocyte count |
| II Indicators of activation of the immune system: | – Lymphocytes<br>– Neopterin<br>– Immunoglobulins (IgA, IgE, IgG, IgM)<br>– Serum complement (Total, C3)<br>– Circulating immune complexes<br>– Circulating antitissue antibodies |
| III Cytokines: | – IFN-γ<br>– MCP-1 (monocyte chemotactic protein-1)<br>– IL-2<br>– IL-6 |
| IV Indicators of autoimmunity: | – Antibodies to Hsps (heat shock proteins)<br>– Antibodies to oxLDL<br>– AECA (anti-endothelial cell antibodies)<br>– CAMs (cellular adhesion molecules) |

### *C-reactive protein*

CRP is the most widely studied APR, produced in the liver, by an inducible activity of interleukin-6. It expresses both proinflammatory and antiinflammatory effects. The proinflammatory effects include: binding to phosphocholine of the pathogen and host; complement system activation; binding to phagocyte cells; initiating of opsonisation; forming cell phagocytosis and lysis; recognizing of autogene substances released from the damaged tissue; proinflammatory cytokines and monocyte tissue factor induction;

ICAM and VCAM induction in endothelial cells. Anti-inflammmatory effects of CRP are as follows: by inhibition of L-selectin synthesis it prevents the adhesion of neutrophyls to endothelium; it inhibits superoxide production by neutrophyls; it stimulates the synthesis of IL-1 receptor antagonists in mononuclear cells. However, the net effect of CRP is antiinflammatory one.

Interleukin 6 is the major inductor of the liver production of CRP whose increase in unstable angina predicts a worse early outcome (12). The potential usefulness of CRP in clinical practice lies in its high predictive value for long term cardiovascular morbidity and mortality in patients with stable angina, as well as for coronary artery disease (13) in an apparently healthy population (11, 14). Increased concentrations of CRP have been associated with a series of risk factors such as diabetes mellitus, hypertension, metabolic syndrome, smoking, obesity, hormone replacement therapy, chronic infections and inflammation. All of them may lead to endothelial activation and/or dysfunction followed by production of a number of inflammatory markers. It is documented that physical activity, weight loss, and treatment with statins or fibrates decreases high sensitive CRP (15).

In patients with stable coronary artery disease and those with acute coronary syndrome, CRP may predict recurrent events and mortality independently of cardiac troponin levels and after adjustment for other prognostic factors (16). In the Women's Health Study (17), a concentration above 3 mg/L showed almost the same prognostic value for event-free survival as the presence the of the metabolic syndrome, and the concentrations of CRP and the total cholesterol/high-density lipoprotein cholesterol (HDL-C) were the only independent predictors of cardiovascular events after adjustment for traditional risk factors. It has also been proved that CRP may be used as a risk factor in women with concentrations of low-density lipoprotein cholesterol (LDL-C) below 130 mg/dL (18). In the Atherosclerotic Risk in Communities (ARIC) Study (19) the relative risk of coronary artery disease after adjusting for risk factors was 1.72 for CRP concentrations above 3.0 mg/L. Similar results were obtained in some other studies (20), suggesting that CRP is an additional measure for estimating the risk of coronary artery disease. CRP currently appears to be the most promising biological marker, although there is still controversy regarding its use in clinical practice.

CRP has also been considered as a therapeutic target. Different classes of statins (atorvastatin, lovastatin, cerivastatin and simvastatin) lower CRP concentrations through mechanisms other than the effects on lipid concentrations (21–25). Thus, the patients who receive statins seem to have a better prognosis even when they have similar concentrations of LDL-C (24). However, the usefulness of hs-CRP as a therapeutic goal and as a parameter for monitoring the response to drugs such as statins, has not been fully established.

*Indicators of immune system activation*

Indicators of immune system activation include lymphocytes, neopterin, immunoglobulins (IgA, IgG, IgM), total serum complement, circulating immune complexes, and circulating antitissue antibodies. T lymphocytes are found in a large number of human atheroscleroc plaques. T-helper (CD4) cells predominate over suppressor (CD8) cells and B lymphocytes (26). In vitro, T lymphocytes from patients with unstable angina, but not those from patients with stable angina or normal controls, proliferated in response to autologous proteins from coronary plaques and/or to oxidized low-density lipoproteins (27).

An increase in the level of circulating cytotoxic T lymphocytes was found in patients with ischemic heart disease. Their number is higher in patients with unstable than in those with stable angina (28). Lymphocytes of patients with unstable angina are immunologically activated and produce soluble factors which may allow their interaction with endothelial cells in areas of inflammation (29). The increment of circulating CD3+/DR+ cells was associated with a better outcome (28).

In acute coronary syndromes an increase in circulating activated lymphocytes and neutrophils, as well as their inflammatory markers and monocyte adhesion molecules, has been documented (8). Increased concentration of interleukin 6 (IL-6) has been found in patients with severe unstable angina (8), and has been shown to be a potent predictor of a poor short-term outcome (30). Interferon-γ (INF-γ) and monocyte, chemotactic protein 1 (MCP-1) were found significantly higher in patients with unstable angina in comparison with both stable angina and control group. Patients with unstable angina and positive troponin T had higher concentrations of INF-γ and MCP-1 than those with negative troponin T (8). Increased INF-γ and MCP-1 in patients with unstable angina, especially in those with elevated concentrations of troponin T, suggests that these cytokines are probably related to myocardial cell damage or to plaque rupture and thrombus formation.

*Neopterin*

Neopterin is a by product of activated monocytes/macrophages stimulated with interferon-γ. Also other cells, such as human umbilical vein endothelial cells or cultured kidney epithelial cells may produce neopterin upon stimulation with INF-γ, but in a smaller amount than the macrophages (31). Studies in patients affected by the syndrome of Mendelian showed that some other stimuli are able to induce neopterin

synthesis in vivo (32). Once produced, neopterin activates the translocation of nuclear factor κB (NFκB) to the nucleus, which in turn upregulates proinflammatory genes such as interleukin-6 and tumor necrosis factor-α. The overall result is an increase in the inflammatory tone within the vascular wall (33). Elevated neopterin levels were found in systemic inflammatory and infectious diseases, malignant tumor diseases and in allograft rejection episodes. In neourological and in cardiovascular diseases cellular immune activation indicated by increased neopterin production was also found (31).

Although a neopterin increase was not observed in patients with stable coronary artery disease (34), serum neopterin is an independent predictor of major adverse coronary events in patients with chronic stable angina pectoris (35). Increased neopterin is associated with the presence of vulnerable or disrupted atheromatous plaques and represents a marker of increased risk of further events in patients with acute coronary syndrome (36). An elevated plasma neopterin level identifies patients at long-term risk of death or recurrent acute coronary events after an acute coronary syndrome. Intensive statin therapy significantly attenuates the risk of recurrent events in those patients (37). Women with unstable angina had significantly higher neopterin concentrations than those without events (33). In our study (unpublished data) neopterin was significantly increased in both the patients with unstable angina and those with stable angina in comparison with healthy subjects. Significantly lower baseline neopterin values were found in patients with acute myocardial infarction who died due to cardiac death during follow up.

On the basis of the above discussed results, neopterin seems to be »still a forgotten biomarker«, but another new inflammatory marker fractalkin (CX3CL1) could be a promissing marker in the future for the detection and prevention of ischemic heart disease. Namely, fractalkine is the only member of the CX3C family of chemokines with the unique property of being also an adhesion molecule. It is expressed on endothelial cells activated by proinflammatory cytokines, such as IFN-γ and α, and its receptor, CX3CR1, is expressed on the T lymphocytes, as well as macrophages. Double knockout CX3CR1-/-, apoE-/-mice present less atherosclerotic lesions and reduced macrophage accumulation than apoE-/- mice expressing the fractalkine receptor (38). It was observed that cells expressing CX3CR1-IM allele have impaired adhesive and chemotactic functions, and CX3CR1 I249 has been reported to be associated with a reduced risk of coronary artery disease (39).

*Pregnancy associated plasma protein A*

Pregnancy associated plasma protein A is a zinc-binding enzyme which belongs to the metalloproteinase superfamily. Measurement of PAPP-A is useful for screening the fetus for Down syndrom in the first three months of pregnancy, because decreased circulating levels of this enzyme are associated with abnormal placental function (40). Besides placenta, many other tissues may produce PAPP-A, but at much lower concentrations than those found during pregnancy. Since PAPP-A is a specific protein whose substrate is insulin growth factor (IGF) it appears as a growth modulator in local proliferative responses to IGF, playing the role of IGF in the pathogenesis of atherosclerosis (41). This action would give PAPP-A an important role in the progression of atherosclerosis.

At first, Bayes-Genis et al. (42) found high levels of PAPP-A in the cells and the extracellular matrix of the plaques that showed rupture or erosion compared to stable atherosclerotic plaques. Several studies have shown that circulating concentrations of PAPP-A are higher in patients with acute coronary syndrome than in those with stable angina and healthy subjects (42). The others found a correlation between PAPP-A levels and troponin (43). By comparisson receiver operating characteristics (ROS) curves for PAPP-A and CRP in patients with acute myocardial infarction higher area under the curve was found for PAPP-A (42). Contrary to those results, Dominguez-Rodriguez et al. (44) found no differences between the PAPP-A concentration in patients with ST-elevation myocardial infarction and controls. The possible relationship of PAPP-A with other cardiovascular risk factors has been studied but with contradictory results (9). The available evidence suggest that measuring plasma concentrations of PAPP-A may be used as a marker of unstable atherosclerotic plaque and have prognostic value in patients with acute coronary syndrome.

## Apoptotic Markers

In the heart, apoptosis occurs both during cardiovascular development and in some pathological conditions (45). Apoptotic cell death is documented in the vascular wall of atherosclerotic vessels and in myocytes. It occurs concomitantly with necrosis in the infarcted and reperfused myocardium, in the end-stage failing heart, in postinfarction left ventricular remodeling, in diabetes, and during the regression of hypertrophy (46). Ischemia/reperfusion leading to myocyte cell death has been reported as either necrotic or apoptotic or a combination of both. Ischemia itself may result in reversible injury or irreversible one. In irreversible injury, caspase-3 activation and PARP cleavage were present after the ischemic period, lamin breakdown mainly occurred during reperfusion, and DNA fragmentation indicating the completion of the apoptotic process was evident at late reperfusion. In reversible ischemic injury all of these processes were absent. In experiments with long-term ischemia DNA fragmentation is absent, suggesting that

ischemia triggers the apoptotic cascade and that during reperfusion the process of cell suicide is completed (47). It is also documented that prolonged ischemia associated with acidosis lead to apoptosis of cardiac myocytes and vascular cells. Ischemia is associated with both hypoxia and increased glycolysis and lactic acid production which decreases pH. Hypoxia-induced cell death is mediated by BNIP3, a member of the Bcl-2 family of proteins, whose expression is induced during chronic hypoxia, and acidosis is required to activate the death pathway (48).

Apoptosis may also occur in atherosclerotic coronary arteries, and the significance of apoptosis depends on the stage of the plaque, localization and the cell types involved. In initial lesions, only a few cells undergo apoptosis. In the neointima apoptotic cells are present in all stages of atherosclerosis, and in intraplaque small vessels. In advanced lesions, many cells are found to undergo apoptosis. Fas/APO-1 is documented in foam cells whose source are macrophages or SMC. Bcl-2 was detected in Fas/APO-1 expressing plaques. Further, CD3-positive T lymphocytes found around foam cells have been found to express Fas/APO-1 (49). Both macrophages and SMC undergo apoptosis in atherosclerotic plaques but also endothelial and blood borne cells (49). SMC become susceptible to apoptosis in the early stages of atherosclerosis because they increase different proapoptotic factors. Also, SMC may be killed by the activated macrophages. Since most of the interstitial collagen fibres, important for the tensile strength of the fibrous cap are produced by SMC, the loss of SMC may attenuate plaque stability. On the other hand, the apoptosis of macrophages may be beneficial for plaque stability if apoptotic bodies were removed. Unscavenged apoptotic cells activate thrombin, which could induce intraplaque thrombosis (50).

An important role in vascular remodeling belongs to reactive oxygen species which participate in vascular SMC growth and migration; modulation of the endothelial function, including endothelium-dependent relaxation and expression of proinflammatory phenotype; and modification of the extracellular matrix (51). Reactive oxygen species, particulary hydrogen peroxide, may also induce apoptotic cell death. Nitric oxide (NO) is the second significant inductor of apoptosis in the vascular wall. Cytokines including TNF-$\alpha$, IL-1$\beta$ and IFN-$\gamma$ may be highly pro-apoptotic through the induction of inducible nitric oxyde synthase (iNOS) and the subsequent production of NO. Cells induced to express iNOS can produce micromolar amounts of NO which in the presence of superoxide can produce peroxynitrite required for NO-mediated apoptosis. Caspase-3 has been shown to be inactivated by peroxynitrite in vitro. Co-generation of NO and superoxide within the mitochondria can lead to the cytochrome c release with limited effect on cytosolic caspase, so the net effect would be increased apoptosis (46).

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is present in stable atherosclerotic lesions, is increased in vulnerable plaques, and is found to be colocalized with CD3 cells and oxLDL. Serum levels of soluble TRAIL are reduced significantly in patients with unstable angina in comparison to patients with stable angina and healthy subjects (52). Contrary, a significant increase in the concentrations of sCD95 and interleukin-1$\beta$ converting enzyme were found in the serum of patients with unstable angina as compared to those with stable angina. Both sCD95 and sCD95L may be detected changed in the serum of subjects at high cardiovascular risk (53, 54), and are considered to be the markers of active atherosclerosis (55). The results of our study (unpublished data) showed that in patients with different clinical expression of ischemic heart disease caspase-3 activity, Fas/APO-1 as well as Bcl-2 were found to be significantly changed.

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