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Proteomic Studies in Coronary Atherosclerosis

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ABSTRACT

Proteomic studies have made a significant contribution to the study of the pathogenesis of cardiovascular diseases, creating the basis for the development of new potential biomarkers for assessing the risk of developing diseases and their complications. We analyzed the main foreign and domestic publications over the past 15 years using the PubMed/Medline and RSCI/elibrary.ru databases and summarized the available data on proteomic studies in the field of atherosclerotic cardiovascular diseases and coronary atherosclerosis. In this literature review, priority was given to studies on the search for new proteomic biomarkers of coronary atherosclerosis, including proteomic markers of unstable atherosclerotic plaques. The data from our own proteomic studies on potential biomarkers of coronary atherosclerosis are presented.

Keywords: atherosclerosis, proteomics, mass spectrometry, biomarkers; macrophages

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Протеомные исследования при коронарном атеросклерозе

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РЕЗЮМЕ

Протеомные исследования внесли существенный вклад в изучение патогенеза сердечно-сосудистых заболеваний, создавая основу для разработки новых потенциальных биомаркеров оценки риска развития заболеваний и их осложнений.

Цель исследования: обобщить имеющиеся данные о протеомных исследованиях в области сердечно-сосудистых заболеваний атеросклеротического генеза и коронарного атеросклероза. Проведен анализ основных зарубежных и отечественных источников преимущественно за последние 15 лет по базам данных PubMed/Medline, РИНЦ/ELIBRARY.RU. Приоритет был отдан исследованиям по поиску новых протеомных биомаркеров коронарного атеросклероза, в том числе протеомных маркеров нестабильной атеросклеротической

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бляшки. Приведены данные собственных протеомных исследований потенциальных биомаркеров в области коронарного атеросклероза.

Ключевые слова: атеросклероз, протеомика, масс-спектрометрия, биомаркеры, макрофаги

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

In the modern world, the study of early atherosclerosis and its complications is one of the most relevant areas of scientific research on cardiovascular diseases (CVD). Proteomic studies have made a significant contribution to the study of the pathogenesis of CVD, creating the basis for the development of new potential biomarkers for assessing the risk of developing diseases and their complications.

Atherosclerosis is a chronic progressive inflammatory disease of large and medium-sized arteries characterized by the formation of atherosclerotic plaques. Atherosclerotic lesions occur as a result of abnormal lipid retention in the intima of the arterial wall, which leads to the production of cytokines and inflammatory mediators by vascular cells, attracting circulating monocytes to the lesion site [1, 2]. The progressive accumulation of lipids, lipoproteins, and inflammatory cells leads to the formation of a fatty strip, which subsequently evolves into an extensive lesion and atheroma [3]. The pathology develops slowly, and, as a rule, the symptoms of atherosclerosis do not appear for several years. However, the continued growth of the plaque reduces the lumen of blood vessels to the point where obstruction of coronary blood flow begins, which leads to stable angina pectoris. By itself, it rarely causes death, but in obstructive and non-obstructive atherosclerotic plaques, erosion or rupture can occur, resulting in clinical complications, such as ischemia, myocardial infarction, and death from cardiovascular events [4].

Modern research methods enhance the understanding of the processes responsible for the progression of atherosclerotic plaques. Thanks to the currently available proteomic methods, new potential biomarkers have been identified for predicting the risks of developing adverse cardiovascular events [5–7].

PROTEOMIC STUDIES OF ATHEROSCLEROTIC PLAQUES

To study the involvement of proteins in the pathological process of coronary atherosclerosis, it is important to investigate the specific relationships between proteins in the coronary arteries, protein expression, and concentration. The proteomic profile of the vascular wall in coronary atherosclerosis can help identify possible diagnostically significant protein structures or potential biomarkers of the disease and develop new approaches to the diagnosis of coronary atherosclerosis and its complications.

In the first large-scale proteomic study of human coronary artery proteins and coronary atherosclerotic plaques, 806 differentially expressed proteins were identified. Some of them were involved in the development of atherosclerosis, while others might be involved in the progression of the disease. All of them were divided into four groups: 1) extracellular matrix proteins, 2) lipid-binding proteins and proteins related to metabolism, 3) proteins related to inflammation, and 4) phagocytic ligands and receptors of apoptotic cells [8].

From the molecular biology perspective, coronary artery disease can be defined as a community of thousands of proteins that collectively alter cellular processes and lead to a characteristic remodeling of the local coronary artery environment. To characterize the proteome of human coronary arteries, samples of coronary arteries in two autopsy cases (men aged 64 and 69 years), divided into 20 segments, were studied using proteomic research methods. One hundred seventy-four differentially expressed proteins were detected in pathological and healthy intima.

The molecular functions of these proteins primarily included: binding (41.47%), catalytic activity (33.24%), transporter activity (9.41%), and structural molecular activity (7.06%) [9].

Serum amyloid P-component (SAP) is an acute phase protein which plays a significant role in the biological processes of the cardiovascular system, such as inflammation and fibrosis. Increased SAP expression was observed in hemorrhagic atherosclerotic plaques of the carotid arteries compared to fibrous plaques [10]. Annexin 5 is found in the vascular endothelium and has anti-inflammatory, anticoagulant, and anti-apoptotic effects due to binding of phosphatidylserine molecules [7]. It has been shown that the level of annexin 5 in the blood increases significantly after the destruction of the atherosclerotic plaque [11]. In a later study, at the stage of unstable atherosclerotic plaques of the coronary arteries, increased levels of SAP and annexin 5 were noted [12].

The human arterial proteome and features associated with early atherosclerosis of the coronary arteries and aortic samples (sectional material from 100 people aged 15–55 years, 200 arterial samples) were studied using mass spectrometry. Significant differences were found in the prevalence of mitochondrial protein, tumor necrosis factor α, insulin receptor, PPAR-α and -γ between coronary and aortic samples, between atherosclerotic and healthy tissues. It was shown that some biomarkers of tissue proteins indicating early atherosclerosis predict anatomically defined coronary atherosclerosis, thereby confirming the possibility of using human tissue proteomics for clinical and diagnostic purposes. The authors concluded that the human arterial proteome can be considered as a complex network, the architectural features of which vary significantly depending on the anatomical position and the presence or absence of atherosclerosis [13].

Cyclin dependent kinases (CDK) are serine / threonine kinases which phosphorylate the corresponding amino acid residues in proteins. There are 11 known CDKs, each of which is activated by one or more cyclins and other similar molecules after reaching their critical concentration. CDK9 is activated by cyclins T1, T2a, T2b, and K. With a decrease in intracellular cyclin concentration, reversible CDK inactivation occurs. In the study, patients with coronary atherosclerosis showed high concentrations of CDK9 compared to the control group. In addition, high enzyme values correlated with a high content of CD14 and monocytes/macrophages in the atherosclerotic focus. The authors suggest that CDK9 may be a potential biomarker of atherosclerotic inflammation [14].

In a proteomic study of atherosclerotic plaque homogenates obtained during endarterectomy in patients with carotid artery atherosclerosis, the authors identified a group of 33 proteins differentially expressing stable and unstable plaques. A steady increase in ferritin, SOD2, and fibrinogen (fragment D) and a decrease in the levels of glutathione transferase and SOD3 were found in unstable plaques. The mass spectrometry data were confirmed by Western blot analysis. The functional importance of the different isoforms of SOD is not yet clear. Increased fibrinogen levels (fragment D) may contribute to the instability of atherosclerotic plaques. In addition, positive correlations were obtained between the level of ferritin in the blood and in the homogenates of atherosclerotic plaques, which allowed the authors to consider ferritin as a potential marker of atherosclerosis progression [15].

Similar results were obtained by another group of scientists. When comparing the proteomic profiles of homogenates of stable and unstable atherosclerotic plaques obtained from the same person, it was found that the unstable plaques had high concentrations of ferritin and fibrinogen, while the stable atherosclerotic plaques were dominated by apoE, actin and L-lactate dehydrogenase B. The identified proteins, according to the authors, may be potential markers of complications of atherosclerotic lesions [16].

Four hundred sixty-three proteins were studied in the analysis of atherosclerotic plaques and blood plasma of patients with atherosclerosis (n = 34) who underwent carotid endarterectomy (n = 14), compared to the protein profile of healthy volunteers. Consistently high levels of thrombospondin-1, a protein that regulates cell interactions with one other and with the extracellular matrix, and vitamin D-binding protein were obtained. The data were obtained by liquid chromatography and mass spectrometry and confirmed by Western blot analysis [17].

A complex of proteomic research methods revealed 118 proteins differentially expressed in fibrous and hemorrhagic plaques. This allowed the authors to identify three biological processes associated with atherosclerosis (platelet degranulation, vascular autophagy, and negative regulation of fibrinolysis). The data from proteomic studies made it possible to identify new biomarkers (calponin-1, DJ-1, vascular

endothelial growth factor, and procollagen C protease enhancer) of plaque vulnerability [10].

In the study of cancer biomarkers, it was found that vascular smooth muscle cells have different and unusual morphology in the atherosclerotic plaque, which correlates with the proliferative state of the cells. Proteomic analysis revealed proteins associated with the formation of atherosclerosis, including mimecan (osteoglycin), Ras-1 suppressor protein (RSUP-1) and cathepsin D, which were simultaneously identified as biomarkers of cancerous tumors. In this case, the expression of mimecan and RSUP-1 was suppressed in the atherosclerotic plaque, while the expression of cathepsin D was increased [17]. Earlier studies also identified a decrease in osteoglycin expression in hemorrhagic atherosclerotic plaques, which, according to the authors, can lead to plaque instability [10]. On the other hand, there are studies with the opposite point of view. It was shown that the concentration of osteoglycin in the blood of patients with coronary artery disease increases. However, in patients with complex coronary lesions, its level was reduced, and it was suggested that osteoglycin plays a role in stabilizing coronary plaques [18]. In a study examining the prognostic value of certain biomarker proteins in patients with coronary artery disease, circulating osteoglycin (mimecan), whose expression is elevated in vulnerable atherosclerotic plaques, was named a promising biomarker of adverse cardiovascular events [19]. A study performed using proteomic methods confirmed the high content of mimecan in samples of stable fibrous and unstable necrotic – dystrophic atherosclerotic plaques in patients with coronary atherosclerosis [12].

Endothelial cells form a metabolically active barrier between the vascular lumen and the vascular wall. Oxidative stress and modifications of tubulin, a component of microtubules of endothelial cells, destabilize vascular integrity and increase permeability, leading to increased cardiovascular risk [20]. In rabbits with hyperlipidemia and atherosclerotic changes, the regulation of tropomyosin, actin, and keratin proteins in the tissues of the carotid artery and the middle cerebral artery was increased [21]. Mutations in the tropomyosin 1 gene can cause hereditary cardiomyopathy, left ventricular hypertrophy, or impaired diastolic function in the absence of hypertension and aortic stenosis [22].

In patients with coronary atherosclerosis, the proteomic profile of stable atherosclerotic plaques of the coronary arteries showed a significant increase in the content of proteins: actin, tropomyosin, vimentin, keratin, tubulin, and microfibrils of associated glycoprotein 4 (MAGP-4) [12].

Human serum albumin (HSA) is the main protein in human blood plasma. It has been demonstrated that a low concentration of HSA in the blood is a prognostic factor of atherosclerosis in blood vessels, regardless of traditional risk factors in patients with HIV infection. In addition, HSA has been shown to be associated with markers of systemic inflammation and hypercoagulation (interleukin 6, tumor necrosis factor α, C-reactive protein, fibrinogen, and D-dimer). The pathophysiological mechanism underlying this association is the ability of HSA to bind many ligands, including proatherogenic ones, thereby preventing their contribution to oxidative stress [23]. The unstable atherosclerotic plaque is characterized by overexpression of various proatherogenic factors and ligands, which possibly leads to the transfer of HSA from blood plasma to atherosclerotic foci. A proteomic study of unstable atherosclerotic plaques of the necrotic – dystrophic type confirmed the increased content of HSA and fibrinogen [12].

PROTEOMICS OF MACROPHAGES IN ATHEROSCLEROSIS

The most important components of the atherosclerotic plaque are immune cells, primarily macrophages [24]. Plaque macrophages are formed mainly due to the differentiation of circulating monocytes recruited from the bloodstream. These monocytes infiltrate the arterial wall during the transmigration process, which involves adhesion molecules and chemotactic factors. In addition, it has recently been shown that the resident population of macrophages can also be maintained by local proliferation. Macrophages are involved in all stages of atherosclerotic lesion, from onset to progression and rupture. In addition, macrophages contribute to inflammation, lipid accumulation, formation of the necrotic nucleus, and degradation of fibrous thickening leading to plaque rupture. However, macrophages are a heterogeneous and plastic population. It has recently been shown that they can also participate in the stabilization of atherosclerotic plaques and even contribute to their regression [24].

MACROPHAGE PHENOTYPES

In response to stimuli from the microenvironment, such as growth factors, cytokines, and chemokines, macrophages differentiate into different phenotypes. Previously, macrophages were divided into classical (M1) and alternative (M2) macrophages with

proinflammatory characteristics. In particular, cytokines, which include tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ) and granulocyte – macrophage colony-stimulating factor (GM-CSF), or bacterial products, such as lipopolysaccharides (LPS), direct macrophages towards the classical phenotype. Alternative macrophages are induced by cytokines, such as interleukins IL-4 and IL-13 or macrophage colony stimulating factor (M-CSF).

M1 macrophages produce high levels of proinflammatory cytokines, including IL-12, IL-23, IL-6, IL-1b, IL-8, and TNFα, and low levels of the anti-inflammatory cytokine IL-10. In addition, they exhibit increased microbicidal activity and release large amounts of reactive oxygen species and nitrogen-containing radicals.

In contrast to proinflammatory M1 macrophages, high levels of transforming growth factor β (TGFβ) and low levels of IL-12 and IL-23 characterize the M2 macrophage phenotype. M2 macrophages express the mannose receptor (CD206) in large numbers and promote wound healing through the process of efferocytosis, matrix remodeling, and fibroblast recruitment [25, 26]. The division of macrophages into M1 and M2 is based on *in vitro* observations and reflects extreme manifestations within a wide range of different macrophage phenotypes. Currently, this classification seems to be an overly simplified view of the complex heterogeneity of macrophage phenotypes, especially in the atherosclerotic plaque, where a diverse microenvironment is involved in the transformation of macrophages towards either the M1 or M2 phenotype. First of all, there are four subtypes in the M2 phenotype. M2a macrophages are induced by IL-4 or IL-13. They stimulate cell growth and tissue repair, and they are characterized by high endocytosis activity and increased expression of CC chemokine ligands – CCL17, CCL18, and CCL22. M2b macrophages are induced by immune complexes, IL-1b, and toll-like receptors and also modulate immune and inflammatory responses. In comparison with other M2 macrophages, they demonstrate the ability to produce both anti-inflammatory and proinflammatory cytokines, such as IL-10, IL-6, IL-1b, and TNFα [27].

The M2c phenotype or inactivated macrophages are induced by TGFβ, IL-10, and glucocorticoids. They secrete CCL16 and CCL18 in large amount and show high capacity for efferocytosis [28]. Finally, M2d macrophages obtained after stimulation with agonists of the toll-like receptor and the adenosine A2A receptor produce vascular endothelial growth factor in high concentrations and IL-12 and TNFα in low concentrations. In addition, unlike other M2 phenotypes, this subgroup does not show high levels of the CD206 receptor [29].

Various subpopulations of macrophages associated with the presence of hemoglobin and erythrocytes have been identified in hemorrhagic areas of human atherosclerotic plaque. M(Hb) macrophages express high levels of CD206 and CD163, the scavenger receptor for the hemoglobin/haptoglobin complex, which is necessary for effective hemoglobin clearance after intracellular hemorrhage [30]. After the digestion of red blood cells, the released heme group can stimulate the polarization of macrophages into the Mhem phenotype, followed by the activation of transcription factor 1. This activation leads to the expression of heme oxygenase-1 (HO-1), the liver X-receptor (LXR)-α and the ATP-binding cassette transporter ABCA1, which demonstrates an atheroprotective effect and prevents the formation of foam cells [31, 32].

THE ROLE OF MACROPHAGE PHENOTYPES IN THE ATHEROSCLEROTIC PLAQUE

The development of the atherosclerotic plaque, as well as its activity, is associated with an increase in the total number of resident macrophages in the plaque. In particular, the number of M1 and M2 macrophages increases with plaque growth, and the total number of macrophages in an unstable plaque is greater than in a stable one [33, 34]. Since each macrophage phenotype exhibits different properties and demonstrates different functions, the predominance of a particular phenotype can have a strong effect on plaque development, stabilization, or regression. It has been shown that macrophages localized in the shoulder of the plaque (a site prone to rupture) mainly exhibit a proinflammatory phenotype and express M1 markers, whereas macrophages located in the fibrous cap express both M1 and M2 markers [33]. Thus, if M1 macrophages located in the fibrous cap are involved in destabilizing the plaque by producing MMP, M2 macrophages can partially stop such a destabilizing effect by releasing profibrotic factors, such as fibronectin, insulin-like growth factor, and TGFβ, which helps stabilize the plaque [35]. Therefore, there is predominance of M2 macrophages in stable plaques. In contrast, M1 macrophages predominate in rupture-prone plaques [34–36].

Atherosclerotic plaques from the human carotid artery were analyzed using real-time PCR and

Western blotting. When analyzing the cellular contents and distribution of macrophages of the M1 and M2 phenotypes, it was shown that in stable plaques, the expression of CD68 was 3 times lower, the expression of ABCA1 was 2.7 times lower, and the expression of CD206 (M2 marker) was 2 times higher than in unstable plaques. In addition, it was shown that M2 macrophages in stable plaques were found in relatively large numbers (42 ± 5% of the total population of macrophages), while in unstable plaques, they accounted for only 23 ± 3% [37]. Based on this, it is possible to hypothesize that the balance between M1 and M2 macrophages, as well as their distribution in the plaque, can strongly influence the fate of atherosclerotic lesions.

A comprehensive study of various macrophage phenotypes and their prevalence in the plaque may be important for predicting a clinical outcome and preventing fatal events in CVD. Since proteins are the main active factors in most biological processes, a proteomic profile can become an effective tool for identifying complex molecular pathways in multifactorial diseases, including atherosclerosis.

PROTEOMIC ANALYSIS OF MACROPHAGES IN THE ATHEROSCLEROTIC PLAQUE

The atherosclerotic plaque is a complex structure consisting of several cell types with different phenotypes. The nature of changes in the plaque strongly depends on intercellular interactions. Thanks to proteomic analysis of the plaque, it is possible to obtain a wide range of proteins involved in the development of atherosclerosis. The presence of a large number of proteins produced by macrophages in the atherosclerotic plaque also confirms the important role of these cells.

Analysis of 35 atherosclerotic plaques from human coronary arteries using direct tissue proteomics with LC-MS/TMS allowed for the identification of 806 proteins, which provided the first full-scale proteomic map of human coronary atherosclerotic plaques. It has been shown that among these proteins, annexin I is expressed in resident macrophages in the inner lining of the vascular wall, which exhibit the foam cell phenotype. [8]. In addition, in this study, the authors showed that the method of direct tissue proteomics is comparable to laser capture microdissection, and using this method it is possible to determine the absolute number of specific cytokines and growth factors in coronary arteries found in low concentrations.

A study was conducted using LC-MS/TMS analysis on extracts of stable areas and areas after rupture from newly isolated plaques from the human carotid artery. During the analysis, several proteins and biological pathways associated with plaque rupture were identified, such as plaque loss of basement membrane proteins, extracellular proteolysis, inflammation, and decreased cell matrix adhesion, which were confirmed in ruptured plaque extracts from the human carotid artery [38].

Macrophages play a crucial role in the development and progression of atherosclerotic plaques. They represent a complex heterogeneous population of several phenotypes, which are characterized by different and often opposite functions. The ability to determine the overall profile of each phenotype is an attractive goal for developing therapeutic strategies aimed at stopping disease progression and stimulating regression. Proteomics provides an effective tool that includes various high-performance and constantly evolving methods that can help understand the diversity of cells present in the atherosclerotic plaque and their behavior. The proteome is a rich source of potential biomarkers that may be useful for characterizing the progression of atherosclerosis and identifying diagnostic and therapeutic targets aimed at plaque stabilization and/or regression [39].

THE MALDI METHOD AND THE PROTEOMIC PROFILE OF BLOOD SERUM IN CORONARY ATHEROSCLEROSIS

In our study of the proteomic profile of blood serum in coronary atherosclerosis, we used the traditional matrix-activated laser desorption/ionization (MALDI) method based on the NCBI database with protein separation by 2D electrophoresis. Blood serum samples from patients with coronary artery disease and coronary atherosclerosis and the “no coronary artery disease” group were examined. The study of proteins was carried out on pools (mixtures) of blood sera. Groups of protein fractions were identified, the protein content of which varied by more than 1.5 times between the experiment and the control ($p < 0.05$). Most of the proteins, the level of which varies in serum samples of patients with coronary atherosclerosis, can be attributed to acute phase proteins and transport proteins: ceruloplasmin, transthyretin, retinol-binding protein 4, hemopexin and proteins − components of the complement system C3, C4, and C9. The level of kininogen and transcription regulators, zinc finger protein 133, and

B-cell CLL/lymphoma 6 member B protein, also decreased in the blood of patients. An increase in the level of the following proteins in the blood serum pool of patients with coronary atherosclerosis was revealed: hemopexin, transthyretin, retinol-binding protein 4, complement system proteins C4, C9, and C3 (chain B) (Table 1) [40].

It is known that in atherosclerosis and CVD, the complement system is activated [41]. Our study revealed an increase in the content of complement components C3 (chain B), C4, C9 and a decrease in the level of complement component C3 (chain C) in the serum pool of patients with coronary atherosclerosis. A comparison of the positions of these proteins on the gel with their theoretical molecular weight indicates that, apparently, we discovered some isoforms of C3 (C chain). To date, there is no data on the relationship between serum concentrations of various isoforms of component C3 chains and atherosclerosis.

Table 1

Mass Spectrometry Identification of Serum Proteins [40]

Spot No.	NCBI Id	Protein name	Mass, kDa	pI	sc, %	score	Δ
1.1	gi|386789	hemopexin	51512	6.57	40	70	+1.7
1.2	gi|386789	hemopexin	51512	6.57	26	72	+2
1.3	gi|386789	hemopexin	51512	6.57	26	74	+1.6
1.4	gi|386789	hemopexin	51512	6.57	28	78	+5.7
2.1	gi|180249	ceruloplasmin	97637	5.29	29	97	–1.6
2.2	gi|47125416	ceruloplasmin	24668	8.52	57	113	–3.8
2.3	gi|47125416	ceruloplasmin	24668	8.52	52	69	–2.0
3.1	gi|545478558	zinc finger protein 133 isoform f	70201	9.43	33	70	–11
3.2	gi|545478558	zinc finger protein 133 isoform f	70201	9.43	31	68	–10
4	gi|62898910	kininogen 1	47823	6.29	36	74	–1.8
5.1	gi|78101271	complement component C3c, chain C	39463	4.79	58	102	–3.5
5.2	gi|78101271	complement component C3c, chain C	39463	4.79	43	79	–2.4
6	gi|2258128	complement component 9	61728	5.42	26	91	+2
7	gi|78101270	complement component C3c, chain B	21482	5.84	45	114	+2.9
8	gi|401871713	complement component C4 chain C	33052	6.37	45	70	+1.4
9.1	gi|212374952	transthyretin, variant V20s, chain A	13741	5.35	89	176	+4.6
9.2	gi|377656323	transthyretin, chain A	12869	5,33	81	82	–2.7
9.3	gi|377656323	transthyretin, chain A	12869	5.33	81	83	+3.6
9.4	gi|2098255	transthyretin, chain A	13829	5.35	59	67	+3.7
10	gi|305677614	retinol binding protein 4, chain A	20018	5.24	77	105	+9

Note: Δ – the change in the concentration of proteins in the serum of patients with coronary atherosclerosis relative to the control, sc – sequence coverage.

Transthyretin and retinol-binding protein are functionally interacting proteins that form the vitamin A transport complex. In our study, three isoforms of transthyretin (9.1, 9.3, and 9.4) were detected, the concentration of which increased in the serum of patients, and one isoform (9.2) with a reduced concentration (Table 1). The sum of staining intensities of all the detected isoforms showed that the total concentration of transthyretin increased in the sera of sick people. Isoforms 9.2 and 9.4 have the same isoelectric point, and, apparently, 9.4 is a monomer, and 9.2 is a multimeric form. Thus, the monomeric form of transthyretin predominated in the sera of patients with atherosclerosis. Transthyretin is synthesized in the liver. In plasma, it is in the form of a homotetramer weighing 55 kDa and consisting of subunits weighing 13.8 kDa; it provides transport of thyroxine and retinol. Improper assembly of the tetramer, including due to point mutations, can lead to the formation of amyloid fibrils, which often occurs in affected arteries [42]. The concentration of the trimeric form of transthyretin is negatively correlated with the risk of developing CVD [43].

In plasma, transthyretin binds to retinol-binding protein, forming a complex that functions as a vitamin A transport system. The concentration of retinol-binding protein 4 is associated with cardiovascular risk factors associated with insulin resistance and coronary artery disease, therefore, this protein can be a marker of metabolic complications, atherosclerosis

and is used to assess coronary artery disease [44]. In addition, it is known that in patients with coronary atherosclerosis, increased levels of retinol-binding protein 4 correlate with the severity of the disease [45].

Hemopexin, an acute phase glycoprotein that binds hemoglobin and free heme, protects the body from possible oxidative damage. It is known that iron accumulates in atherosclerotic plaques and affected areas of the arteries and in a catalytically active form can cause proatherogenic events, such as the production of reactive oxygen species and lipid peroxidation [46]. Therefore, some researchers consider hemopexin as a protective protein in this process, although its role in atherosclerosis is not fully understood [47]. We have revealed an increase in the concentration of four isoforms of hemopexin in the sera of patients with coronary atherosclerosis [40].

Ceruloplasmin is a specific copper-containing plasma glycoprotein that belongs to acute phase proteins. Ceruloplasmin has pro- and anti-inflammatory properties, so its role in atherosclerosis is controversial. There is evidence that a reduced level of ceruloplasmin may be an unfavorable prognostic sign in patients with coronary artery disease when combined with high concentrations of C-reactive protein, a well-known marker of the acute phase of inflammation [48]. However, there are studies that associate high levels of ceruloplasmin with heart failure [49]. Analysis of proteins decreasing in the blood serum pool of patients with coronary atherosclerosis showed a decrease in the level of one isoform and two fragments of ceruloplasmin [50].

THE MRM METHOD IN STUDIES OF THE RELATIONSHIP OF SERUM PROTEIN CONCENTRATIONS WITH UNSTABLE ATHEROSCLEROTIC PLAQUE IN CORONARY ATHEROSCLEROSIS

The MALDI method of identifying proteins by their peptide maps has become a true standard in proteomic research. However, further improvements in methods and instruments have made it possible to analyze complex mixtures and achieve higher accuracy. Modern quantitative proteomic analysis, used for the identification and determination of biological molecules based on mass spectrometry, is a method with accurate quantitative simultaneous determination of a large number of proteins in various biological samples. Multiple reaction monitoring (MRM) with internal standard peptides labeled with stable isotopes is the most widely used method for the absolute quantitative analysis of target proteins in the field of proteomics [51].

We examined blood serum samples from 40 men, group 1 (St) – patients who, according to the histological analysis, had only stable atherosclerotic plaques (AP) in their samples; group 2 (Unst) – patients who, according to the histological analysis, had only unstable APs in their samples [52]. Protein concentrations in the blood serum samples were measured using the PeptiQuant Plus Proteomics Kit (Cambridge Isotope Laboratories, USA), the proteins were identified by the MRM method on an ultra-high resolution triple quadrupole time-of-flight mass spectrometer with electrospray ionization combined with a high-performance liquid chromatograph. As a result of the comparative analysis, proteins were isolated, the concentration of which was statistically significantly different in the studied groups ($p < 0.05$) (Table 2).

Serum samples of patients with unstable APs exhibited increased concentrations of the proteins attractin, complement factor H, fibrinogen, and fibulin-1, as well as reduced levels of proteins involved in the development of the inflammatory process and the body's immune response, such as ceruloplasmin, hemopexin, haptoglobin, afamin, complement components (C3, C7, C9), and complement factor B. At the same time, there was a reduced level of proteins involved in the coagulation cascade and fibrinolysis and proteins functionally related to them (α-2-antiplasmin, α-2-macroglobulin, heparin cofactor 2, coagulation factor XII, prothrombin, plasminogen, PAI-1, vitronectin). Multifactorial logistic regression analysis confirmed the association of instability with the concentration of attractin (OR = 1.045; $p = 0.027$), afamin (OR = 0.988; $p = 0.001$), hemopexin (OR = 0.997; $p = 0.020$), haptoglobin (OR = 0.967; $p = 0.001$), and components of the complement system. In addition, multifactorial logistic regression analysis showed an association of instability with an increased concentration of fibulin-1 (OR = 1.008; $p = 0.05$) in patients with unstable APs.

PROTEOMIC PROFILE OF ATHEROSCLEROTIC PLAQUE TISSUE AT DIFFERENT STAGES OF DEVELOPMENT IN CORONARY ATHEROSCLEROSIS

To study the involvement of proteins in the pathological process of coronary atherosclerosis, it is important to investigate the specific relationships

between proteins at different stages of development of atherosclerotic lesions in the coronary arteries. In addition to changes in known lipid and inflammatory molecules, certain proteins can influence the development of atherosclerotic lesions into unstable plaques. Temporal analysis of the proteomic profile of the vascular wall in coronary atherosclerosis can help identify possible diagnostically significant protein structures or potential biomarkers of the disease and develop new approaches to the diagnosis of coronary atherosclerosis and its complications.

We examined tissue samples of atherosclerotic plaques containing intima media of the coronary arteries. All samples were obtained from patients who had undergone coronary artery endarterectomy during the operation for intraoperative indications. Samples from male patients were taken for the study with similar clinical characteristics. According to the results of the morphological and histological analysis, all samples were classified as stable or unstable atherosclerotic plaques. A mix of atherosclerotic plaque homogenates at different stages of development were prepared for proteomic analysis.: 1) mix of stable atherosclerotic plaque homogenates at the stage of lipidosis and fibrosis (StL), 2) mix of stable atherosclerotic plaque homogenates at the stage of fibrosis and calcification (StF), 3) mix of unstable atherosclerotic plaque homogenates of necrotic – dystrophic type (Unst). Moreover, the proteomic profiles of every atherosclerotic plaque were determined. The proteins were identified by MALDI mass spectrometry using tryptic mass maps with the Mascot search algorithm. To find differences, a quantitative comparison criterion was used for the difference of at least 1.5 times between the average values of the staining intensity of protein spots (in relative fluorescence units (RFU)) in the groups corresponding to 3 stages (Table 3).

Table 2

Quantitative Mass Spectrometry Identification of Proteins in the Blood, *M* ± *SD* [52]

No.	Protein name	Protein concentration, fmol/μl		*p*
		Group (St)	Group (Unst)	
1	serum albumin	374440.00 ± 61793.83	354465.00 ± 58076.57	0.140
2	ceruloplasmin	1891.77 ± 511.66	1646.48 ± 418.60	0.021
3	α-1-acid glycoprotein	18027.10 ± 7298.18	13287.65 ± 4678.42	0.001
4	α-1- antichymotrypsin	6224.75 ± 3299.37	4545.25 ± 2367.32	0.011
5	α-1- antitrypsin	27696.0 ± 7929.29	23672.0 ± 9887.34	0.048
6	hemoglobin (subunit α)	4785.9 ± 2342.02	4204.15 ± 2608.95	0.297
7	haptoglobin	589.55 ± 261.55	479.60 ± 194.18	0.036
8	hemopexin	1973.6 ± 247.48	1756.55 ± 310.65	0.001
9	serotransferrin	19999.50 ± 3002.74	18329.50 ± 3243.45	0.019
10	retinol-binding protein 4	1237.08 ± 287.36	1372.42 ± 413.33	0.093
11	transthyretin	510.13 ± 179.43	640.23 ± 456.87	0.098
12	afamin	330.12 ± 117.85	264.59 ± 73.53	0.004
13	apolipoprotein A-I	21096.25 ± 6127.08	21626.0 ± 3662.74	0.640
14	apolipoprotein B-100	276.24 ± 79.53	211.04 ± 68.84	0.0001
15	apolipoprotein C-I	5019.2 ± 1251.40	5069.6 ± 1353.80	0.863
16	apolipoprotein L1	590.28 ± 158.45	501.25 ± 200.51	0.031
17	complement component C1q (subunit B)	75.86 ± 31.96	67.07 ± 17.17	0.129
18	complement component C1q (subunit C)	117.84 ± 36.25	120.22 ± 35.42	0.768
19	complement component C1r	230.49 ± 51.37	210.20 ± 70.84	0.147
20	complement component C1s	47.18 ± 10.83	48.99 ± 22.84	0.652
21	complement component C3	590.51 ± 137.97	516.46 ± 139.39	0.019
22	complement component C7	73.23 ± 19.38	61.94 ± 11.18	0.002
23	complement component C9	167.05 ± 66.10	138.93 ± 56.85	0.045
24	complement factor B	4951.7 ± 1358.16	4215.7 ± 1135.39	0.010
25	complement factor H	530.54 ± 79.29	577.37 ± 84.59	0.014
26	attractin	48.43 ± 9.97	55.17 ± 17.14	0.035
27	fibrinogen, α-chain	143.55 ± 4.79	261.0 ± 21.88	0.001
28	fibrinogen, γ-chain	55.3 ± 32.9	113.04 ± 72.14	0.001
29	fibulin-1	660.54 ± 98.04	713.33 ± 131.49	0.045

Table 3

Comparison of the Amount of Protein for Pools / Individual Gels in Atherosclerotic Plaque Homogenates, RFU, ×10^5 [53]

No.	Protein name	MIX / INDIVID		
		StL (n = 5)	StF (n = 5)	Unst (n = 5)
1–3	serum albumin	4.3 / 5.2	12.3 / 7.7	46.3 / 22.6
4–6	vimentin	101 / 12.9	2.4 / 5	4.1 / 13.2
7	tubulin (β chain)	2.5 / 7.6	1.4 / 3.1	1.1 / 4.3
8–10	actin (α cardiac muscle 1) actin (aortic smooth muscle)	84 / 98.3*	29.2 / 43.5	33.4 / 32.1
11	actin (cytoplasmic)	91.3 / 92.6	18.4 / 53.1	37.7 / 33.4
12–14	fibrinogen (β chain)	1.3 /–	3.2 / 19.2	2.9 / 331.2
15	tropomyosin (β chain)	40.3 / 37.9	2 / 17.0	2 / 12.9
16	tropomyosin (α1 chain)	– / 7.8	–/ 2.7	– / 4
17–19	microfibril-associated glycoprotein 4	22.4 / 39.4	4.5 / 54.9	3.2 / 26.7
20	mimekan	26.5 / 44.0	126.5 / 63.7	55.4 / 49.3
21	annexin A5	2.8 / 8	0.7 / 16.1	2.2 / 8.4
22	keratin (type I cytoskeletal 9)	6.4 / 23.8*	–/ 7.5	1.7 / 2
23–24	serum amyloid (P-component)	25.3 / 40	5.9 / 48.8	22.2 / 61
25	peroxiredoxin-2	– / 1	– / 4.7	– / 2.5

Note. MIX – average data for pools of plaque homogenates; INDIVID – average data of individual plaque homogenate gels; maximum values are shown in bold; * statistically significant differences, $p < 0.05$.

A comparison of individual atherosclerotic plaque gels showed the existence of significant individual differences in the staining intensity of specific spots between plaque samples within the same stage. As the plaque develops from stable at the stage of lipoidosis and fibrosis to unstable one, these differences increase. This fact did not allow us to detect statistically significant differences between the groups of samples at different stages in most cases. Isoforms of the same protein, differing only in point pI, were grouped together. Summary data on the comparison of the amount of protein (RFU) in the corresponding spots in the StL, StF, and Unst groups are shown in Table 3. At the stage of lipoidosis and fibrosis of stable atherosclerotic plaques, the content of cytoskeletal proteins increased: actin, tubulin, tropomyosin, keratin, and vimentin. At the stage of fibrosis and calcification of stable atherosclerotic plaques, a significant increase in the level of proteins responsible for regulating cell migration and proliferation and involved in redox homeostasis of cells was found: microfibril-associated glycoprotein-4, mimecan, annexin A5, and peroxiredoxin-2. Unstable atherosclerotic plaques (necrotic – dystrophic type) were characterized by high levels of serum albumin, fibrinogen, serum amyloid (P-component), and a maximum content of vimentin [53].

The results of these studies represent a potential proteomic platform for further study of plaque instability in coronary atherosclerosis. The potential role of the studied proteins in the development of coronary atherosclerosis, as well as their prognostic value as biomarkers of atherosclerotic plaque instability, should be the subject of further research.

CONCLUSION

Attempts to clarify the molecular mechanisms underlying cardiovascular diseases have been made repeatedly, however, these diseases still remain some of the leading causes of death worldwide. Atherosclerosis is the morphological basis of coronary artery disease and its complications. Cardiovascular proteomics is a new field in which significant progress has been made over the past few years in identifying new candidate biomarkers for the diagnosis and obtaining information on the molecular pathophysiology of cardiovascular diseases. The human blood plasma proteome reflects the physiological state of the cardiovascular system and has been used for decades to study plasma biomarkers in a standard analysis designed to diagnose and monitor cardiovascular diseases.

Diagnosing patients with high-risk atherosclerotic plaques before clinical manifestations remains a difficult task and requires an improved approach to predicting the onset of symptoms. The development of proteomic technologies has made it possible to

analyze proteins associated with the development of the disease. These changes reflect the molecular and cellular mechanisms and may make it possible to predict the dynamics of the disease.

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