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CIRCULATING LEVELS OF POTENTIAL MARKERS OF ISCHEMIC STROKE IN PATIENTS WITH THE DIFFERENT FORMS OF ATRIAL FIBRILLATION AND CHRONIC HEART FAILURE

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Atrial fibrillation (AF) is the most common abnormal type of heart rhythm (cardiac arrhythmia), which is considered the leading cause of stroke. There have been limited studies on the prognostic markers for atrial disease and AF-associated ischemic stroke, despite the high demand for this procedure in daily clinical practice to monitor disease course and assess risk of stroke in patients with AF and chronic heart failure (CHF). Thus, the aim of the present study was to evaluate the levels of serum biomarkers related to ischemic stroke in CHF patients with the different forms of AF. Forty-six patients with various types of AF (paroxysmal, persistent and permanent) with or without ischemic stroke were enrolled in the study, 36 clinically healthy donors served as a control. The levels of inducible nitric oxide synthase (iNOS), vascular endothelial growth factor (VEGF) and angiostatins (AS) were evaluated by western blot analysis in the serum. The levels of active matrix metalloproteinases (MMPs) were analysed by gelatin zymography. Elevated levels of iNOS were shown in patients with all AF forms as compared with control, but iNOS levels in post-ischemic patients were significantly higher than that in paroxysmal AF individuals. However, the levels of VEGF and AS did not differ from the baseline value in patients with paroxysmal AF, while dramatic increase of their contents was shown in post-stroke patients with persistent and permanent types of AF. Elevated active MMP-9 levels were shown to be associated with the diagnosis of all AF forms, regardless of the occurrence of stroke. Taken together, our findings demonstrate that tested proteins can be considered as valuable biomarkers of AF forms transformation and potentially useful for ischemic stroke risk stratification in patients with AF and CHF. Observed changes in regulatory protein levels may expand our understanding of pathological roles of endothelial function dysregulation, disrupted angiogenesis balance and abnormal tissue remodeling in AF and associated ischemic events.

K e y w o r d s: atrial fibrillation, ischemic stroke, biomarkers, VEGF, iNOS, angiostatins, MMP-9.

Atrial fibrillation (AF) is the most common type of cardiac arrhythmia, which affects 2.5–3.5% of the population worldwide [1]. AF is the major cause of mortality and morbidity, mainly related to embolic events. Due to cardiac rhythm irregularity, blood flow through the heart becomes turbulent and has a high chance of forming a blood clot, which can ultimately dislodge and cause a stroke. If left uncontrolled, AF can lead to serious and life-threatening complications, including stroke or heart failure. According to its temporal pattern, AF is classified as paroxysmal, persistent and permanent (chronic). In paroxysmal type of AF, irregular heartbeat may last from thirty seconds to a week and often passes without symptoms, while permanent AF is characterized by consistent abnormal heart rhythm. All these forms of AF are associated with an increased risk of thrombotic events, heart failure, dementia and stroke [2]. Thus, the main negative impact of AF is the high risk of cardioembolic stroke that remains high at all clinical forms of AF. In the management of patients with AF, an easy-to-use transition from population assessment and traditional risk factors to individual stroke risk reclassification tools is needed for timely preventive and therapeutic decisions [3].

According to existing recommendations, the management of patients with AF should take into account comorbidity [4]. AF and chronic heart failure (CHF) occur frequently, the simultaneous presence of these two conditions is common. AF and CHF share common risk factors (e.g., hypertension) and may promote each other [5]. CHF may influence the development of AF due to fluid retention, electrolyte imbalance, and inducing procoagulant state [6]. Currently, the concept of atrial disease is being formed. Detection of subclinical atrial disease aims to prevent worsening of the disease course, including AF, stroke and CHF [7]. Treatment of AF and its associated complications causes a significant and growing economic burden. In patients with AF, a model including selected blood markers could reveal new insights on residual silent stroke risk and justify unscheduled brain MRI. However, knowledge gaps remain in the understanding of pathogenetic mechanisms of these conditions, and their elucidation is necessary to develop appropriate preventive measures, diagnostics and management for patients with concomitant AF and CHF. For investigation of molecular mechanisms that underlie AF and verification of the disease that led to its development, atrial tissue specimens are often used as an endomyocardial biopsy taken from patients with a prolonged or persistent form of arrhythmia. Thus, there is a need in seeking adequate peripheral biomarkers of AF progression for further testing [8].

Activation of the coagulation system as a usual consequence of AF development has a significant proinflammatory impact on atrial myocardium, which may promote tissue remodeling and atrial fibrosis, thus contributing to rhythm disruption and AF aggravation [9, 10]. Atrial fibrosis is generally accepted to be a key factor leading to the development of AF [11]. Hypoxia, which is developed in atrial tissue due to fibrosis, activates multiple signaling pathways regulated by hypoxia inducible factor-1 (HIF-1). Under ischemic or fibrotic conditions, HIF-1 has been shown to play a key role in the upregulation of vascular endothelial growth factor (VEGF) expression [12]. VEGF is a major *in vivo* mitogen for endothelial cells that plays an essential role in activating neovascularization in ischemic tissues to provide an adaptive mechanism for restoring blood supply interrupted due to ischemic impact [13]. Therefore, VEGF can be considered as a plausible biomarker candidate for the evaluation of AF persistence.

Hypoxia, inflammation and myocardial fibrosis are interconnected and implicated in hypercoagulation development and endothelial dysfunction in patients with various types of AF [14, 15]. Inducible nitric oxide synthase (iNOS) upregulation may greatly contribute to AF pathogenesis through involvement in tissue remodeling and cardiomyocyte apoptosis, and thus can be used as a biomarker of endothelial dysfunction in AF and CHF patients [16]. Numerous experimental and clinical studies have confirmed the importance of matrix metalloproteases (MMPs) in atrial remodeling and myocardial fibrotic lesions in patients with both AF and CHF [17, 18, 19]. Among various extracellular matrix protein degradation products produced by MMP and several other proteases (e.g., macrophage metalloelastase, neutrophil elastase or plasmin), proteolytic fragments of glycoprotein plasminogen, known as angiostatins (AS), attract special attention because they have been found to be one of the most potent physiological inhibitors of endothelial cell activity and suppressors of angiogenesis [20]. AS comprise a group of plasminogen-derived polypeptides, which differ by various amount of kringle (K) domains (K1-3, K1-4, K1-4.5, K5) determining their biological activity [21]. Extended data have been accumulated that AS are implicated in the complex pathomechanisms of cardiovascular diseases by disrupting local angiogenic balance that leads to inhibition of reparative angiogenesis in ischemic tissues and sustaining fibrosis [22, 23]. Although there are several lines of evidence that increased circulating levels of AS are correlated with severity of different cardiovascular diseases [24, 25], the possible link between AS levels and AF progression has yet to be elucidated. It is believed that the levels of MMPs or possible bioactive MMP-induced degradation products in the circulation may have a prognostic value and serve as reliable markers able to estimate the current condition of the heart tissue.

Thus, we aimed here to investigate the relationship between circulating biomarkers (levels of iNOS, VEGF, AS and MMP activity) and ischemic stroke risk in different forms of AF with concomitant CHF.

Materials and Methods

Patients and study population. Forty-six patients with established AF were included in a non-interventional study. The criteria for inclusion in the study were the presence of established AF in accordance with recommendations of the Euro-

pean Society of Cardiology [4] on the background of established coronary heart disease, CHF with preserved ejection fraction (≥50%), the presence of a previous stroke with resumed anticoagulants for more than two weeks or the absence of a previous stroke and age from 65 to 85 years. Exclusion criteria for the patients enrolled in the study were as follows: recent acute myocardial infarction (<6 months), acute coronary syndrome, history of hemorrhagic stroke, previous ischemic stroke within two weeks, hematologic or oncologic diseases, diabetes mellitus, liver diseases, hypothyroidism, severe anemia, GFR<30 ml/min/m^2 and grade 4 obesity.

The study was noninterventional, all patients were previously observed and treated by a cardiologist, neurologist or family doctor. Patients were divided into two groups: (A) patients with a history of ischemic stroke (n = 19), and (B) patients with no history of stroke (n = 27). Subsequently, a subanalysis was carried out with the selection of the following groups: (1) healthy control, (2) non-stroke AF patients, (3) patients with persistent AF and stroke and (4) patients with permanent AF and stroke. The healthy control group consisted of 36 donors with absent AF, CHF, acute thrombotic conditions and comparable in age and gender.

The clinical and demographic characteristics of the patients are presented in the Table.

The allocated groups were comparable in terms of antihypertensive drugs and CHF treatment. In patients with AF and previous ischemic stroke, circulating biomarkers (iNOS, VEGF and AS levels, and MMP activity) were determined in a period of two weeks with renewal of anticoagulant treatment as a term of inpatient observation associated with reducing risks of hemorrhagic transformation and lower acute thrombotic state impact.

Ethical approval. Informed written consent was obtained from all patients before their participation in the study. The investigation conforms to the principles outlined in the latest revision of the Helsinki Declaration. All study procedures and protocols used in the investigation were reviewed and approved by the local ethical committee of Dnipro State Medical University (the initial protocol No. 6, February 7, 2018, last updated March 20, 2023).

Reagents and antibodies. All chemicals were purchased from Sigma Aldrich Co. (St Louis, MO, USA) unless otherwise stated. Mouse monoclonal anti-VEGF antibody was purchased in Invitrogen, USA (cat. no. MA5-12184, used in 1:3,000 dilution). Rabbit anti-iNOS antibody was purchased in Cell Signaling Technology, USA (cat. no. 2982, used in 1:1,000 dilution). Rabbit anti-human angiostatin antibodies were produced and validated as described elsewhere [26]. Antibodies against human serum albumin (HSA) were isolated from sera of rabbits immunized by commercially available HSA purchased from Sigma Aldrich (cat. no. A9511, ≥97% purity) and used as an immunoglobulin G (IgG) fraction isolated by affinity chromatography on protein A-Sepharose. Goat horseradish peroxide (HRP)-conjugated anti-rabbit IgG (used in 1:6,000 dilution) and anti-mouse IgG (used in 1:8,000 dilution) were purchased from Invitrogen, USA (cat. nos. G-21234 and 31430, respectively). All other reagents were of

Table. The clinical and demographic characteristics of the patients according to the history of ischemic stroke

Parameter	Group 1 (n = 19)	Group 2 (n = 27)	P
Males, n (%)	8 (42.1)	12 (42.9)	0.67
Females, n (%)	11 (57.9)	16 (57.1)	0.58
Age, years	78.8 [68.7; 81.7]	76.2 [64.4; 79.2]	0.03
Body Mass Index, kg/m^2	27.1 [25.4; 34.5]	26.9 [25.1; 32.2]	0.28
GFR, ml/min/m^2	64,4 [62.1; 72.8]	66.9 [61.1; 74.3]	0.68
Paroxysmal form of AF, n (%)	0	12 (42.9)	0.04
Persistent form of AF, n (%)	8 (42.1)	7 (25.9)	0.14
Permanent form of AF, n (%)	11 (57.9)	8 (29.6)	0.03
Arterial hypertension, n (%)	16 (84.2)	22 (78.6)	0.61
Anticoagulants, n (%)	19 (100)	20 (74.1)	0.04
Antiplatelet drugs, n (%)	0	7 (25.9)	0.02

analytical grade and were provided by commercial suppliers.

Serum sample preparation. For blood serum sampling, blood was taken by venipuncture from all patients and healthy donors after overnight fasting. Whole blood was allowed to form a clot at room temperature for 60 min, and then centrifuged at 1,500 g for 15 min in a refrigerated centrifuge to separate serum from the clot. Then, serum was immediately transferred into a clean polypropylene tube, transported and stored at –20°C until analysis.

Western blot. Western blot analysis was used to assess the levels of circulating VEGF, iNOS and AS to make an intergroup comparison between groups of patients with different stages of AF. Total protein concentration in serum was evaluated spectrophotometrically using the measurements of absorbance at the wavelengths of 260 and 280 nm as described elsewhere [27]. Serum of healthy donors and AF-CHF patients was diluted in sodium dodecyl sulphate (SDS)-containing Laemmli sample buffer (×5). For analysis of proteins of interest, except AS, sample buffer contained 10% 2-mercaptoethanol. Serum samples were electrophoresed in 10% SDS-PAGE loading 100 μg protein per track. After electrophoresis, serum proteins were transferred onto nitrocellulose membrane (GE Healthcare, Amersham Bioscience, UK, RPN 203D, 0.45 μm pore diameter) by electroblot, and then were blocked in 5% skim milk for 90 min at 37°C. After blocking, the blots were probed with the anti-iNOS, -VEGF, -AS or -HSA antibodies overnight at 4°C. After washing in phosphate buffered saline containing 0.05% Triton X-100 (PBST) five times, each membrane was incubated with correspondent anti-specie HRP-conjugated secondary antibody for 2 h at 37°C. After washing in PBST, specific immunoreactivity was developed by enhanced chemiluminescence with the use of p-coumaric acid, luminol and hydrogen peroxide [28]. The molecular weights of each protein band were determined by comparing their migration with the location of colored markers PageRuler™ Plus Prestained Protein Ladder 10 to 250 kDa (Thermo Scientific, Lithuania, cat. no. 26619) on the nitrocellulose membrane. Each band immunostaining was quantified by measuring optical density values with the use of densitometry software TotalLab TL120 (Nonlinear Inc., USA) and expressed as arbitrary units.

Gelatin zymography (MMP assay). The MMP activity was analyzed by separating serum proteins (50 μg/track) in 8% SDS-PAGE gel copolymerized with gelatin (5 mg/ml). After electrophoresis, the gels were washed twice for 30 min in the ice-cold 2.5% (v/v) Triton X-100 to remove SDS, and then six times for 5 min in ice-cold bi-distilled water. After washing, gels were incubated in developing buffer (50 mM tris-HCl, pH 7.6, containing 0.15 M NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and 0.03% Tween-20) overnight at 37°C. Zymograms were stained with 0.15% Coomassie Brilliant Blue R-250 solution in 25% methanol and 10% acetic acid, and then destained in the same solution lacking Coomassie Blue. The final gels had a uniform blue background except in those regions to which MMPs migrated and cleaved the substrate. Thus, the active MMP forms were identified as transparent bands against the background of blue-stained gelatin. The MMP bands were visualized and quantified densitometrically.

Statistical analysis. The results are presented as mean ± SEM. Analysis of variances (ANOVA) followed by post-hoc Tukey's multiple comparison test was used to verify significant difference between group means. Results of semi-quantitative analysis of western blots and zymograms are presented as "box-and-whiskers" plots. OriginPro software (version 8.6, OriginLab Corporation, Northampton, MA, USA) was used to perform all statistical calculations. *P* value of less than 0.05 was considered significant.

Results

Among 46 patients with AF and CHF, 19 (41.3%) had ischemic stroke. Patients with stroke were older ($P = 0.03$) and had persistent or chronic form of AF. The levels of biomarkers, which may reflect transformation of AF types and serve as indices of stroke risk assessment, were evaluated in serum of enrolled patients and healthy control donors by western blot. As shown in Fig. 1, all AF patients had increased iNOS levels as compared with control group. However, iNOS level in non-stroke patients was 1.76-fold higher than baseline value ($P = 0.025$), while in patients after stroke elevation in iNOS levels was much higher (2.5- and 2.55-folds, $P = 0.003$ and 0.0002 in persistent and permanent AF, respectively). Interestingly, circulating levels of iNOS in patients with more profound forms of AF were higher than that in patients with paroxysmal AF ($P = 0.042$ and 0.03).

The major angiogenic regulator, VEGF, was detected in serum samples as a single polypeptide of about 25 kDa (Fig. 2). Densitometry analysis of blotograms revealed a trend in increasing VEGF levels

in non-stroke patients, but not reaching statistical significance. However, 4.45- and 3.85-fold increase in VEGF levels were shown in post-stroke patients with persistent or permanent AF as compared with control (P = 0.02 and 0.041, respectively).

Circulating AS forms were detected as two immunoreactive polypeptides with molecular weights of 50 and 15 kDa, which corresponds a major AS form K1-4.5 and minor AS variant K5, respectively (Fig. 3). Similarly to VEGF, the most profound changes in AS levels were observed in post-stroke AF patients. In persistent or permanent AF forms, levels of AS K1-4.5 were two- and three-folds higher versus the control value (P = 0.08 and 0.0002, respectively). It is important to note that there was a statistically significant dependence of K1-4.5 increase from progression of AF in post-stroke patients (1.5-fold elevation of K1-4.5 level in persistent AF versus permanent form, P = 0.007).

AS K5 appeared to be almost undetectable in healthy donors and non-stroke patients. However, dramatic overproduction of K5 reaching the peak level was observed in post-stroke patients with chronic AF (122-fold increase vs control, P = 0.026, and 5.8-fold increase vs persistent AF group, P = 0.044).

Gelatin zymography was used to examine MMP activity in the serum of patients and control individuals. The results of MMP assay depicted in Fig. 4 show the presence of both latent (pro-) and active forms of MMPs in the tested samples. Pre-MMP-9 was present in the serum of healthy donors, while little if any active enzyme was detected. Unlike healthy control, all AF patients with or without stroke had enhanced levels of both pro- and active MMP-9 in circulation.

Immunochemical analysis of serum albumin revealed no statistically significant changes in the protein levels between the studied groups of patients (Fig. 5). This result obviously indicates that these pathological conditions have no effect on the protein synthetic function of liver. Therefore, it can be assumed that albumin level can be utilized as a loading control for electrophoretic assays of blood proteins.

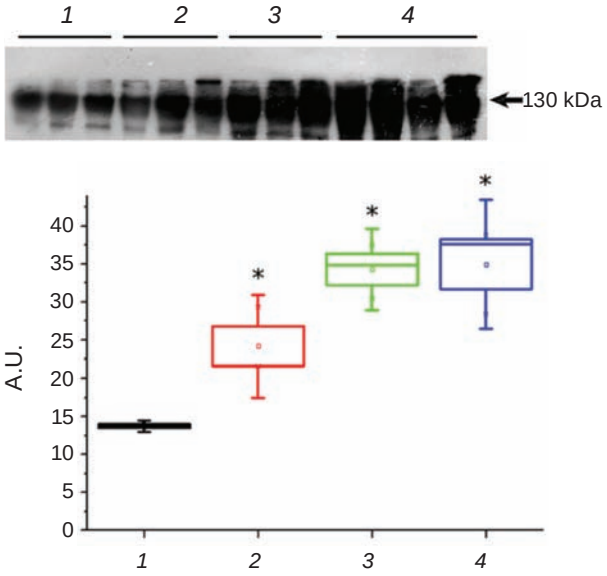

*Fig. 1. The circulating levels of inducible nitric oxide synthase in patients with atrial fibrillation (AF) and chronic heart disease with or without ischemic stroke (typical blotogram and densitometry analysis): (1) healthy control, (2) non-stroke AF patients, (3) patients with persistent AF and stroke, and (4) patients with permanent AF and stroke (*P < 0.05 compared with control (ANOVA) followed by post-hoc Tukey's test); A.U. – arbitrary units*

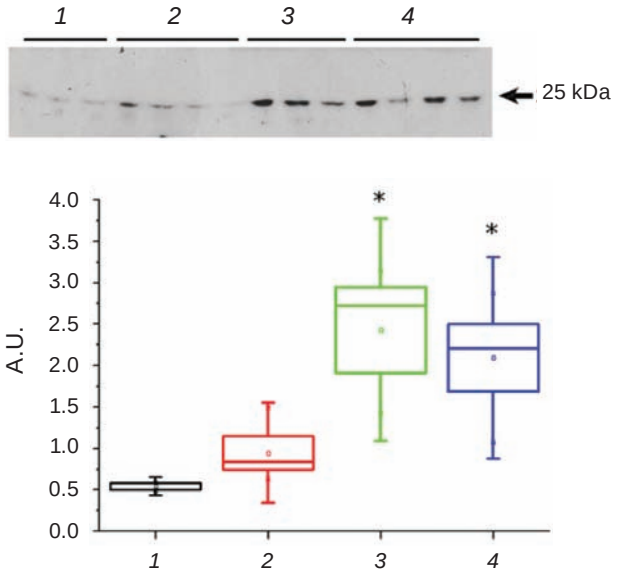

*Fig. 2. The circulating levels of vascular endothelial growth factor in patients with atrial fibrillation (AF) and chronic heart disease with or without ischemic stroke (typical blotogram and densitometry analysis): (1) healthy control, (2) non-stroke AF patients, (3) patients with persistent AF and stroke, and (4) patients with permanent AF and stroke (*P < 0.05 compared with control (ANOVA) followed by post-hoc Tukey's test); A.U. – arbitrary units*

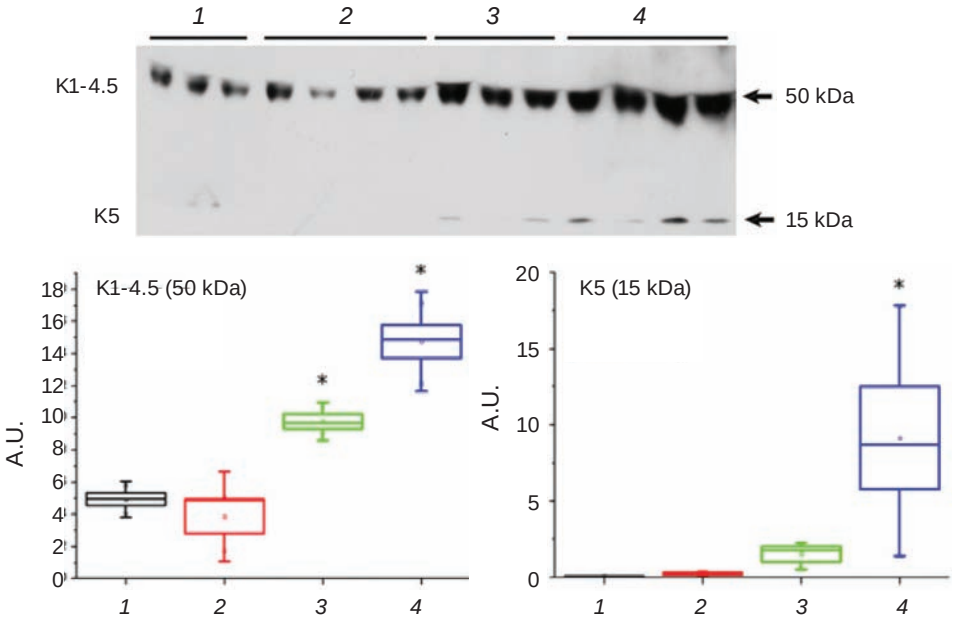

*Fig. 3. The circulating levels of angiostatins polypeptides in patients with atrial fibrillation (AF) and chronic heart disease with or without ischemic stroke (typical blotogram and densitometry analysis): (1) healthy control, (2) non-stroke AF patients, (3) patients with persistent AF and stroke, and (4) patients with permanent AF and stroke (*P < 0.05 compared with control (ANOVA) followed by post-hoc Tukey's test)*

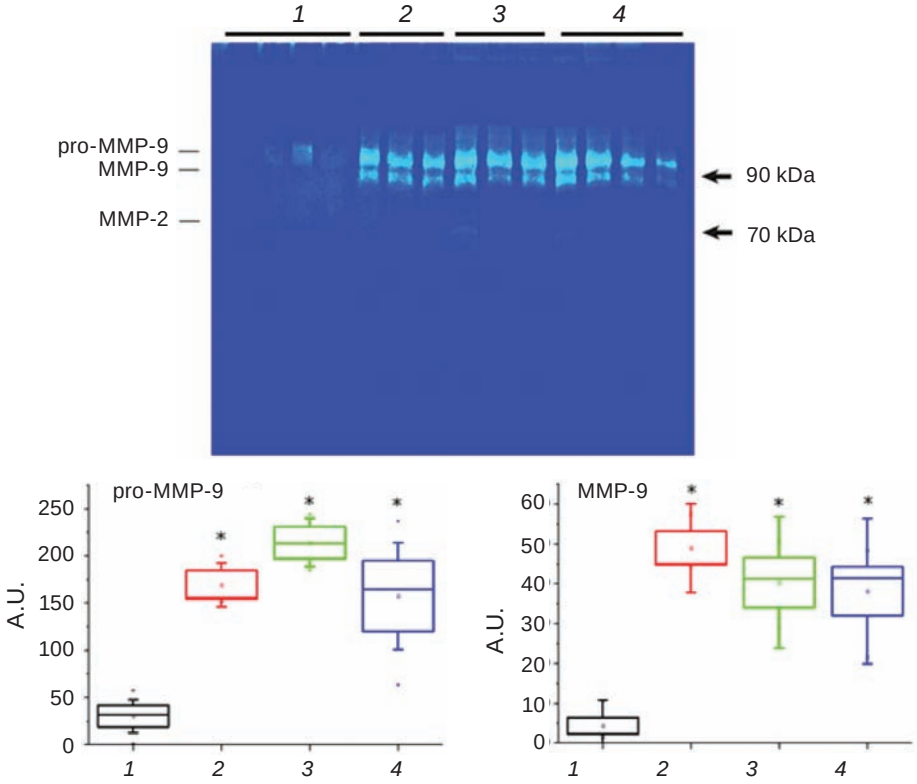

*Fig. 4. The circulating levels of matrix metalloproteases (MMPs) in patients with atrial fibrillation (AF) and chronic heart disease with or without ischemic stroke (typical zymogram and densitometry analysis): (1) healthy control, (2) non-stroke AF patients, (3) patients with persistent AF and stroke, and (4) patients with permanent AF and stroke (*P < 0.05 compared with control (ANOVA) followed by post-hoc Tukey's test); A.U. – arbitrary units*

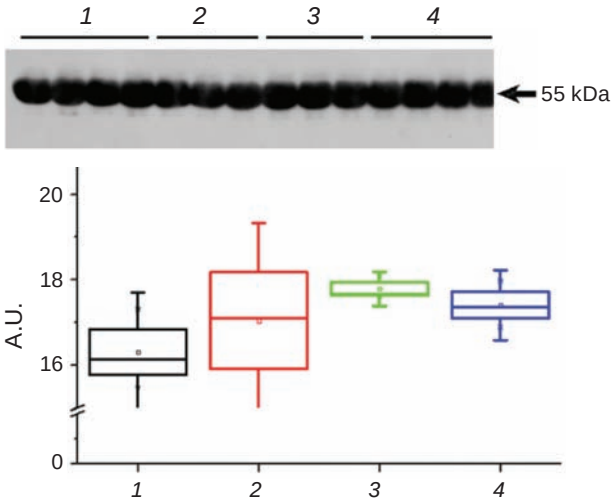

*Fig. 5. The circulating levels of serum albumin in patients with atrial fibrillation (AF) and chronic heart disease with or without ischemic stroke (typical blotogram and densitometry analysis): (1) healthy control, (2) non-stroke AF patients, (3) patients with persistent AF and stroke, and (4) patients with permanent AF and stroke (*P < 0.05 compared with control (ANOVA) followed by post-hoc Tukey's test); A.U. – arbitrary units*

Discussion

In the present pilot study, we investigated the relationship between circulating levels of four biomarkers, which reflect the extent of endothelial dysfunction, angiogenesis, tissue remodeling/fibrosis and different types of AF and assessed their predictive value in AF-associated ischemic stroke. It is generally accepted that AF frequently co-exists with atrial abnormalities, such as impaired myocyte function, fibrosis, atrial mechanical dysfunction and endothelial dysfunction. Even in individuals with difficult-to-detects forms of AF, once AF develops, the dysrhythmia causes contractile dysfunction and stasis, which further increases the risk of thromboembolism. Over time, the dysrhythmia causes structural remodeling of the atrium, thereby worsening atrial cardiopathy and increasing the risk of thromboembolism and even stroke [29].

Nitric oxide (NO) plays a critical role in regulating vascular tone and the antithrombotic properties of the endothelium. Reduced nitric oxide synthase (NOS) expression and low NO levels are widely used signs of endothelial dysfunction. This enzyme exists in three isomeric forms (endothelial NOS [eNOS], neuronal NOS [nNOS] and iNOS) [30]. Endothelial cells constitutively express eNOS, while cardiomyocytes produce all three enzyme forms. Despite most studies supporting the view that eNOS expression and NO levels are reduced in AF, overexpression of iNOS has been shown in patients with permanent AF [16]. As is known, iNOS is expressed in the myocardium during pathologic states such as heart failure [31]. iNOS uncoupling in macrophages has been shown to catalyze reactions in immune response and cytotoxicity of macrophages [32] that suggests a potential role for oxidative stress-mediated atrial pathogenesis and AF [33]. The diffuse distribution of iNOS in myocytes has been reported, suggesting a role for modulation of atrial myocyte pathology [34]. Han et al. [16] demonstrated induction of iNOS in the right atrium in patients with permanent AF compared to those with normal sinus rhythm, suggesting that the induction of iNOS was not a function of heart failure but rather of AF itself. Our study showed a significant increase in the level of iNOS in patients not only with persistent and permanent forms of AF but also with a paroxysmal form. This finding supports a role for iNOS contribution for AF substrate in CHF. On the other hand, our study also demonstrated a significant increase in iNOS among patients with AF and ischemic stroke. As is known, iNOS plays a pivotal role in the neuronal injury during the early and late stages of ischemic stroke and mainly produced by microglia, astrocytes, endothelial cells and infiltrating lymphocytes [35]. The activation of iNOS was reported to be increased from 12 h after the onset of ischemic stroke and lasts for one week [36]. After ischemic stroke, iNOS elevates NO levels and causes nitrosation damage within 12 h to 8 days [37]. It should be noted that in our study, the most dramatic elevation in iNOS levels was demonstrated exactly two weeks after ischemic stroke in patients with AF and CHF. Therefore, the obtained results indicate the modulation of NOS-related pathways as a possible therapeutic strategy in AF.

Angiogenesis plays a key role after stroke neurovascular remodeling. The patients with AF have elevated levels of inflammatory cytokines known to promote vascular leak, such as VEGF that highlights inflammation-induced vascular abnormalities as a potential factor in the development and progression of AF [13]. It has been shown earlier that mechanical overload caused by atrial remodeling and fibrosis can upregulate VEGF secretion by cardiac myocytes, and visa verse VEGF has been shown to stimulate

fibrosis within the atrial tissue [38]. The recent data shows that relatively high plasma levels of vascular endothelial growth factor D (VEGF-D) have also been associated with incidence of CHF [39]. Berntsson et al. described that increased VEGF-D concentrations have been associated with AF related ischaemic stroke [40]. However, the results of our study demonstrate that the most pronounced elevation of VEGF levels occurred in post-stroke patients with persistent or permanent forms of AF. Our findings mostly corroborate with the earlier data of Matsuo et al. [41]. The authors have shown that plasma VEGF concentration increases immediately in patients with different types of stroke and remains elevated as compared with healthy control individuals within 90 days after stroke onset. Since VEGF expression strongly correlates with an extent of ischemic injury, it has been assumed that plasma VEGF is an independent predictor of poor functional outcome in cardioembolic infarctions. Therefore, literature data and our observations strongly support the idea that ischemic events in post-stroke patients trigger angiogenic switch depending on ischemia severity, and increasing VEGF dynamics can be considered a meaningful biomarker of stroke risk in AF patients with CHF.

In healthy tissues, angiogenesis is regulated by a balance between positive regulators, such as VEGF and counteracting inhibitors. AS are produced in many tissues and participate in the regulation of the angiogenic balance by inhibiting the proliferation and migration of endothelial cells and inducing apoptosis in endotheliocytes [20]. Although AS polypeptides have been originally discovered as inhibitors of tumor-mediated angiogenesis [42], there is accumulating data suggesting involvement of AS in pathological processes underlying the development of cardiovascular diseases. For example, Matsunaga et al. have confirmed that increased production of AS can be responsible for impaired coronary angiogenesis and insufficient development of the collateral vascular network via decreasing NO production or its bioavailability in coronary heart disease [22]. In addition, AS have been found to counteract VEGF-mediated proangiogenic signaling, so that AS appeared to inhibit collateral growth in ischemic myocardial tissue despite relatively high levels of VEGF [43, 44]. The results of our present study, in which we found higher levels of AS in post-stroke patients with AF than in healthy control or non-stroke AF patients, are consistent with the prior findings. Recently, we have found elevated AS levels in patients with cerebral ischemia associated with AF, while post-insult treatment with cytidine diphosphate-choline (citicoline) improved serum levels of this angiogenesis inhibitor, thus enhancing pro-angiogenic signaling as a beneficial effect of such treatment option [45]. Western blot allowed us to analyse polypeptide composition of AS variants, which includes a major AS (50 kDa) and a minor form (15 kDa). We found for the first time that low molecular AS isoform, which corresponds to the single K5, is produced in post-stroke patients with persistent form of AF and, therefore, can be considered a potential prognostic marker of cerebral ischemia in patients with chronic AF. Heterogeneity of AS spectrum indicates that various AS-generating proteolytic systems of a different cell origin may be activated in this cohort of patients. Currently, information on the AS production in human ischemic brain tissue or other sources and its physiological/pathophysiological role is extremely limited. Earlier, our group established that brain astrocytes may play an essential role in producing AS in nervous tissue [46]. However, other cell types, which are activated during hypoxia and inflammation including platelets, neutrophils, macrophages/microglia, can act as plausible sources of AS formation and may release them into the bloodstream [47, 48, 49]. The revealed significant increase of AS levels among patients with AF and previous stroke may justify further research of these angiostatic polypeptides as sensitive biomarkers of silent strokes.

Enzymes of MMP family are actively involved in hypoxia-induced tissue remodeling. The results of the present study, which demonstrate an increase in the level of the active form of MMP-9 in the blood serum of patients with various forms of AF, are mostly consistent with previously published data of Lewkowicz et al. [50]. The authors have shown that increased activity of MMP-9 contributes to the occurrence and further development of AF and correlates with the intensity of pathological remodeling of atrial tissue and, in particular, thinning of the left atrial wall. Li et al. [51] analysed the content of active MMP-9 in the blood serum of patients with paroxysmal, persistent and permanent forms of AF and established a direct correlation between the activity of this proteinase and the stages of the development of pathology. In addition, the activation of MMP during the development of AF can participate not only in the remodeling processes of ischemic tissues, but also be responsible for the formation of angiostatins

as a mechanism of inhibition of reparative angiogenesis. Therefore, MMPs together with tissue serine proteinases are involved in the degradation of the extracellular matrix and may play an important role in both the induction and inhibition of angiogenesis. Previous studies have shown that a sharp increase in the expression and activity of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) during ischemic processes in the brain, in an experimental model of lipopolysaccharide-induced CNS damage, contributes to the development of brain pathology and compromised blood-brain barrier integrity [52, 53]. Despite the urgency of the problem, there are very few studies focused on the evaluation of the role of follow-up estimation of circulating MMP-9 in acute ischemic stroke patients. Zheng et al. [54] have demonstrated prognostic significance of serum MMP levels, which may help to predict both short-term and long-term mortality in post-stroke patients. Other investigators have established that elevated serum MMP-9 levels can be an important prognostic factor for ischemic stroke because it correlates with volume of infarction and stroke severity and are associated with increased risk of mortality, major disability and cognitive impairments [55]. MMP-9 level was significantly increased after stroke onset with the level correlating with infarct volume, stroke severity and functional outcome. Higher serum MMP-9 levels in the acute phase of ischemic stroke were associated with increased risk of mortality and major disability, suggesting that serum MMP-9 could be an important prognostic factor for ischemic stroke [56]. Gundogdu et al. [57] have recently demonstrated the possibility of a pharmacotherapeutic approach to reduce MMP-2 and -9 protein levels, as well as their proteolytic activity, which stimulates axonal regeneration after peripheral nerve injury. In the CNS tissue, astrocytes and microglial cells are the major producers of MMPs [58]. Keeping in mind that MMPs are largely responsible for the formation of angiostatins and several other angiosuppressive molecules, MMP activity can serve as a relevant drug target to improve proangiogenic signaling [59].

Our study has some limitations that should be considered. A longer period of follow-up biomarker measurements is required for a better understanding of the dynamic behavior of biomarker levels over time. Residual confounding cannot be excluded completely, although the major cardiovascular risk factors were taken into consideration. Further investigations of the influence of possible medication therapy on biomarker levels in larger prospective studies are needed. Nonetheless, strengths of this study include prospective and population-based cohort. Another strength is that all stroke cases and cardioembolic origin were validated and confirmed by neurologists. However, evaluation of adequate markers of neurodegeneration is also required because necrotic cell death from stroke activates a systemic inflammatory response, which also plays a role in the origin of AF.

Conclusions. Our findings demonstrate that iNOS, VEGF and AS can be considered as valuable biomarkers of AF forms transformation and may have important implications for stroke reclassification. However, elevated MMP-9 circulating levels may indicate development of pathological process in atrial tissue and serve as an early biomarker of AF-associated pathological conditions. Observed changes in regulatory protein levels may expand our understanding of pathological roles of endothelial function dysregulation, disrupted angiogenesis balance, abnormal tissue remodeling in AF and associated ischemic events.

Conflct of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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РІВЕНЬ ПОТЕНЦІЙНИХ МАРКЕРІВ ІНСУЛЬТУ В КРОВІ ПАЦІЄНТІВ ІЗ РІЗНИМИ ФОРМАМИ ФІБРИЛЯЦІЇ ПЕРЕДСЕРДЬ ТА ХРОНІЧНОЮ СЕРЦЕВОЮ НЕДОСТАТНІСТЮ

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Фібриляція передсердь (ФП) є найпоширенішим типом аномального серцевого ритму (серцевої аритмії), що вважається основною причиною інсультів. На сьогодні проведено обмежену кількість досліджень прогностичних маркерів захворювання передсердь та ішемічного інсульту, пов'язаного з ФП, попри існуючий високий попит на цю процедуру в повсякденній клінічній практиці, необхідну для моніторингу перебігу захворювання та оцінювання ризику інсульту у пацієнтів з ФП та хронічною серцевою недостатністю (ХСН). Метою цього дослідження було визначити вміст маркерів, асоційованих із ішемічним інсультом, у сироватці крові пацієнтів із ХСН та різними формами ФП. До дослідження було включено 46 пацієнтів із різними типами ФП (пароксизмальною, персистуючою та перманентною) з ішемічним інсультом або без нього. Контролем слугували 36 клінічно здорових донорів. Рівень індуцибельної синтази оксиду азоту (iNOS), фактора росту ендотелію судин (VEGF) і ангіостатинів (AS) визначали методом Вестерн-блот аналізу зразків сироватки крові. Рівень активних матриксних металопротеаз (MMP) оцінювали за допомогою желатинової зимографії. Зростання рівня iNOS показано у пацієнтів із усіма формами ФП порівняно з контролем, але рівень iNOS у крові постішемічних пацієнтів значно перевищував такий у хворих із пароксизмальною формою ФП. Проте у пацієнтів із пароксизмальною ФП рівні VEGF та AS не відрізнялися від базальних значень, тоді як у постінсультних хворих із персистуючим та хронічним типом ФП спостерігалося різке підвищення їх вмісту. Показано, що підвищення рівня MMP-9 асоціюється з усіма діагностованими формами ФП, незалежно від наявності інсульту. Загалом, результати нашої роботи демонструють, що досліджувані протеїни можна розглядати як важливі біомаркери трансформації форм ФП та є потенційно корисними для стратифікації ризику ішемічного інсульту у пацієнтів із ФП і ХСН. Отримані результати щодо зміни рівнів регуляторних протеїнів можуть розширити уявлення стосовно ролі порушення ендотелійної функції, дисбалансу модуляторів ангіогенезу та підсиленого ремоделювання тканин у патогенезі ФП та асоційованих ішемічних станах.

Ключові слова: фібриляція передсердь, ішемічний інсульт, біомаркери, VEGF, iNOS, ангіостатини, MMP-9.

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