Advances in Medical Sciences 64 (2019) 381–387

Contents lists available at ScienceDirect

Advances in Medical Sciences

journal homepage: www.elsevier.com/locate/advms

Original research article

# The potential role of inflammation in cryptogenic stroke

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ARTICLE INFO

*Keywords:*
Cryptogenic stroke
Biomarkers
Interleukin-6
Inflammatory cytokine

ABSTRACT

*Purpose:* The study aimed to identify biomarkers predictive of cryptogenic stroke in patients aged <65.
*Materials and methods:* We investigated 520 patients with ischemic stroke. Out of them we assigned 65 patients to the cryptogenic stroke group (age 54 (47–58), 42% male) and 36 without stroke to the control group (age 53 (47–58), 61% male). In all patients we assessed carotid intima-media thickness (cIMT) and the levels of biomarkers which might be involved in the underlying biological mechanism of ischemic stroke.
*Results:* There were no differences between stroke and control groups in the levels of syndecan 4, resistin, leptin, low-density lipoprotein cholesterol, triglycerides, prothrombin time, or activated partial thromboplastin time. There was no statistically significant difference in cIMT between groups. The level of high-density lipoprotein cholesterol was statistically significantly lower in the cryptogenic stroke group than in the controls (1.1 mmol/L (0.95–1.46) vs 1.37 (1.19–1.6) p = 0.02). Patients in the stroke group had higher levels of N-terminal pro-brain natriuretic peptide (NT-proBNP) (391 pg/ml (107–1249) vs 109 (46–236); p = 0.003), interleukin 6 (2.6 pg/ml (0.8–8.1) vs 0.7 (0.4–1.2) p = 0.002) and asymmetric dimethylarginine (ADMA) (0.44 μmol/L (0.39-0.55) vs 0.36 (0.32-0.4); p = 0.0002) than the control group. In the multivariate analysis Il-6 was the only biomarker statistically significant associated with the occurrence of cryptogenic stroke (odds ratio 1.918, 95% confidence interval 1.029–3.575; p = 0.04).
*Conclusions:* Endothelial dysfunction assessed by increased level of ADMA affects the inflammatory state in patients with cryptogenic stroke. Increase in the inflammatory cytokine IL-6 by 1 pg/ml almost doubles the risk of stroke.

## 1. Introduction

Stroke is one of the main causes of death and disability worldwide. Ischemic stroke is a heterogeneous disease with different mechanisms and etiologies and specific treatments. A substantial proportion of stroke risk remains unexplained. Ultimately stroke is classified as cryptogenic or undetermined when no cause is identified after a thorough study [1,2]. With over 15 million strokes occurring every year worldwide, methods to better identify patients at risk of stroke are needed, as are methods to improve patient diagnosis and prognosis when stroke occurs [3].

Use of blood-based biomarkers is one method that has been evaluated to predict the risk of stroke, diagnose stroke and its causes, predict stroke severity and outcome, and guide prevention therapy. The role of blood biomarkers in ischemic stroke is still being defined [4].

The aim of this study was to identify circulating biomarkers with a potential etiological connection with ischemic stroke i.e. asymmetric dimethylarginine (ADMA), interleukin 6 (IL-6), N-terminal pro-brain natriuretic peptide (NT-proBNP), syndecan 4, adipokines and the N-terminal propeptide of type III procollagen (PIIINP), and to determine which of them are predictive of cryptogenic stroke in patients aged <65.

Endothelial dysfunction could be caused by elevated ADMA, which inhibits the release of endothelium-derived nitric oxide (NO). The definite role of ADMA after acute ischemic stroke still needs to be clarified. On one hand, ADMA might contribute to brain injury by reducting of cerebral blood flow and on the other hand, ADMA might be involved in NO synthase (NOS)-induced oxidative stress and excitotoxic neuronal

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https://doi.org/10.1016/j.advms.2019.06.001
Received 13 November 2018; Accepted 18 June 2019
Available online 28 June 2019
1896-1126/ 

death. Inflammation may also have a role in cryptogenic stroke. Higher plasma levels of tumor necrosis factor alpha (TNF-α), IL-6 and interleukin-1 beta (IL-1β) in acute ischemic stroke were associated predominantly with cardioembolic subtype, whereas lacunar subtype showed statistically significantly lower plasma levels of these cytokines [5,6]. In the study by Licata et al. [7] acute brain stroke patients presented statistically significantly higher plasma levels of cytokines, selectins, adhesion molecules and PAI-1.

B-type natriuretic peptide (BNP) and NT-proBNP are substances that are produced in the heart and are released when the heart is stretched and is working hard to pump blood. Tests for BNP and NT-proBNP measure their levels in the blood in order to detect and evaluate heart failure. However, NT-proBNP levels also proved to be greatly predictive of incident atrial fibrillation (AF) in cryptogenic patients [8,9].

Syndecan 4 is a transmembrane heparin sulfate-carrying glycoprotein that mediates signal transduction pathways activated by growth factors and cell surface receptors, thereby modulating tissue regeneration, angiogenesis, and focal adhesion. The endothelial glycocalyx maintains vascular integrity, and glycocalyx shedding reflects endothelial dysfunction and early atherosclerosis. Syndecan 4 is a component of the glycocalyx, and increased serum levels indicate glycocalyx damage [10]. Circulating syndecan 4 is increased in heart failure patients and in the study by Solbu et al. [11] it was associated with the incidence of myocardial infarction (MI). This suggests a link between endothelial glycocalyx shedding and coronary heart disease. Using syndecan 4 as a risk marker in ischemic stroke requires investigation.

Adipose tissue is now described as an endocrine organ secreting a number of adipokines contributing to the development of inflammation and metabolic imbalance, but also endothelial dysfunction, vascular remodeling, atherosclerosis, and ischemic stroke. Leptin, adiponectin, and resistin are the most studied adipokines which play important roles in the regulation of cardiovascular homeostasis. Leptin and adiponectin have been linked to the development of coronary heart disease and may be involved in the underlying biological mechanism of ischemic stroke [12]. In the brain, adiponectin signaling through its receptors - adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) - directly influences important brain functions such as energy homeostasis, hippocampal neurogenesis, and synaptic plasticity. This signaling may relate to the association between adiponectin and metabolic disease including atherosclerosis, which is a precipitating factor for stroke [13]. The effect of leptin on blood pressure, sympathetic activation, insulin resistance, platelet aggregation, arterial thrombosis, angiogenesis, and inflammatory vascular responses suggests leptin's role in the development of cardiovascular disease and stroke. However, its role as a risk factor for stroke is still debatable [14]. Resistin, a proinflammatory cytokine, is predictive of atherosclerosis and poor clinical outcomes in patients with coronary artery disease and ischemic stroke. These adipokines have been proposed as potential prognostic biomarkers of cardiovascular mortality/morbidity and therapeutic targets in patients with cardiometabolic diseases [12]. PIIINP is an extension peptide of procollagen type III. Elevated PIIINP reflects myocardial remodeling, which is associated with statistically significant left ventricle (LV) dilatation and a persistently depressed LV ejection fraction [15]. In previous studies such as Lee et al. [15] and Agarwal et al. [16], PIIINP has been associated with multiple indicators of cardiac and vascular structure. Agarwal et al. [16] observed a large number of incident cardiovascular events compared to previous studies and reported separate associations for heart failure, myocardial infarction, and stroke, which revealed notable differences between cardiovascular endpoints. Although these biomarkers are not specific to central nervous system tissues, they were shown to be statistically significantly elevated in patients with acute ischemic stroke, and because their role in patients with cryptogenic stroke had not been assessed, we decided to assess their significance in this study.

## 2. Materials and methods

### 2.1. Study population

We prospectively investigated 520 patients with confirmed ischemic stroke. Out of them we assigned 65 patients to the cryptogenic stroke group [age 54 (47–58) years, 42% male] and 36 patients without stroke to the control group [age 53 (47–58) years, 61% male]. All patients in the cryptogenic stroke group had brain CT scans in the emergency room and also brain MRI in the Department of Neurology. In all patients we assessed carotid intima-media thickness (cIMT) using Doppler ultrasound, the function of the heart performing echocardiography, 72 -h monitoring of electrocardiography (ECG) to exclude paroxysmal AF and/or atrial flutter and the levels of selected biomarkers which might be involved in the underlying biological mechanism of ischemic stroke.

The exclusion criteria were as follows: unstable hypertension, atrial fibrillation, hyperthyroidism, pregnancy and breastfeeding, dialysis, cancer, autoimmunological disease, use of cytostatics, immunosuppressive drugs, glucocorticosteroids, or antiretroviral drugs, undergoing a transplant and receiving treatment with a hematogenous preparation during the last 6 months, active infection, alcoholism, addiction to medicines, infection with hepatitis B virus (HBV), hepatitis C virus (HCV) or human immunodeficiency virus (HIV), surgical intervention or serious injury during the last month, vaccination during the last 3 months, and being incapable of giving consent.

All enrolled patients underwent blinded adjudication by cardiologists experienced in adjudication. Detailed clinical, biomarker and imaging data were collected at the time of enrollment and echocardiograms were performed and interpreted by cardiologists blinded to biomarkers analysis. Fasting venous blood samples were drawn in the morning and the obtained serum was frozen at the temperature of −70 °C. Systolic and diastolic arterial pressures were measured using a sphygmomanometer and stethoscope.

### 2.2. Ethical issues

Ethical approval from the Bioethics Commission of the Medical University of Lodz (Poland) was obtained for this study (approval no. RNN/272/16/KE). All methods in this study were performed in accordance with the guidelines and regulations approved by the Bioethics Commission of the Medical University of Lodz (Poland). Written informed consent was obtained from all the patients.

The study was registered at ClinicalTrials.gov – identifier number: NCT03377465 - “Biomarkers, Hemodynamic and Echocardiographic Predictors of Ischemic Strokes and Their Influence on the Course and Prognosis”.

### 2.3. Echocardiography

All patients were examined following a standardized protocol using an ALOKA Alpha 10 Premier (Tokyo, Japan) with a 3–11 MHz probe after inclusion.

Quantitative echocardiography was used following current guidelines. Left ventricular volumes and ejection fraction (EF) were determined by the biplane Simpson method. Left ventricular mass was calculated using the Devereux formula. The early (E) peak velocity was measured from transmittal flow. The early diastolic (E') mitral annular myocardial velocity of the left ventricle septal and lateral walls was recorded from the apical 4-chamber view with pulsed-wave tissue Doppler and results were averaged. The E/E' was calculated as an index of LV filling pressure.

### 2.4. Laboratory tests

Laboratory tests were performed in fasting patients following a minimum 12 -h period after the last meal. At the initial time point of the

study, 19.5 ml of blood was collected with a vacuum blood collection system from the basilic vein into 8.5 mL, 5 mL, and 4 ml clot-activator plastic Vacutainer tubes and into 2 ml Vacutainer tubes containing ethylene diamine tetra acetic (EDTA), for routine laboratory tests. The blood samples were coded and secured immediately after collection: samples for blood chemistry, immunochemistry and hematology tests were delivered to the hospital laboratory. Hematology (peripheral blood count) was performed using an automated 5-diff hematology analyzer, model Beckman Coulter HMX((Brea,California, US), equipped with reagents from the same company, and the biochemical tests using a biochemical analyzer model AU 640, Olympus (Brea, California, US). Plasma glucose was measured by ultraviolet photometry using the enzymatic reactions catalyzed by hexokinase, using Olympus reagent kits (Cat. No. OSR61221, OSR6221). Plasma creatinine was measured by picric acid-based colorimetric kinetic assay (Jaffe's reaction), using Olympus reagent kits (Cat. No. OSR6178).

Triglycerides, total, and high-density lipoprotein (HDL) cholesterol levels were evaluated using the AU680 device (Beckman Coulter Poland, Warsaw, Poland). Low-density lipoprotein cholesterol (LDL-C) concentrations were calculated by Friedewald's formula: LDL-C (mmol/L) = TC - HDL-C - TG/2.2.

### 2.5. Holter ECG

Holter ECG (72-h) was recorded in a 2-channel, 5-electrode paradigm with a GE SEER Light Ambulatory Recorder and analyzed with the GE Marquette MARS Holter system (GE Medical systems, Milwaukee, WI, USA). Electrode placements were below the right and left clavicle, over I5 sin, I4 dx on the right sternal edge and on the lower right chest wall (ground).

### 2.6. Biomarker tests

The blood samples to measure the levels of biomarkers were taken on the 7th day after stroke. The serum samples for measuring biomarkers were centrifuged and pipetted into Eppendorf tubes, and subsequently placed in a freezer (first at -25 °C, then at −70 °C). After the desired number of samples were obtained and their prior thawing (immediately before testing), the biomarker levels were assessed. Enzyme linked-immunosorbent assay (ELISA) tests were conducted for quantitative determination of NT-proBNP (Cloud-Clone Corp, China), resistin (BioVendor, Czech Republic), adiponectin (BioVendor, Czech Republic), leptin (BioVendor, Czech Republic), IL-6 (Gen-Probe, France), ADMA (Immunodiagnostic, Bensheim), PIIINP (Cloud-Clone Corp, China) and syndecan 4 (Cloud-Clone Corp, China) in human serum. The microtiter plates coated with antibody against appropriate protein were incubated with the serum of patients. The time and temperature of incubation depended on the type of analyzed protein: one hour at 25 °C for resistin, leptin and IL-6, two hours at 25 °C for adiponectin and ADMA, one hour at 37 °C for PIIINP, syndecan 4 and NT-proBNP kits. The plates were washed with wash buffer after incubation. Following the wash, plates were incubated with biotin-conjugated antibody against the studied proteins. After incubation (time and temperature were appropriate for type of the protein, according to manufacturers' protocols) plates were washed and incubated with streptavidin-HRP conjugate for 30 min. The next step involved incubation of plates with substrate solution. The reactions were stopped using stop solution and absorbance for each plate was determined at 450 nm.

### 2.7. Measurement of carotid intima-media thickness

Subclinical carotid atherosclerosis was assessed using a carotid artery ultrasound scanner equipped with a 7.5-MHz linear array imaging probe (Aloka ProSound Alpha 6, Hitachi Aloka Medical America, Wallingford, CT, USA). All measurements were performed by the same physician blinded to the subject's clinical status, with the subject in supine position, the head turned away from the side of interest, and the neck extended slightly. The measurement was performed in the longitudinal plan on both the right and left common carotid arteries, about 2–3 cm proximal to the carotid bifurcation. CIMT of the far wall was evaluated manually as the distance between the lumen-intima interface and the media-adventitia interface. Measurements were obtained manually from five contiguous sites at 12 mm intervals bilaterally, and the mean of measurements was used for statistical analysis. An abnormally increased cIMT was defined as a cIMT of the upper 25 percentiles (≥0.71 mm) in the entire study population [17].

### 2.8. Statistical analysis

The STATISTICA 13.1 software package (StatSoft, Poland) was used for analysis. Results were considered statistically significantly if $p < 0.05$. The Shapiro-Wilk test was used to assess the normality of distribution. Data were presented as mean and standard deviation or median and interquartile range (25%–75%), depending on data scale and distribution. To compare the two groups, Student's *t*-test for continuous variables with normal distribution and with homogeneity of variance was used. For data with normal distribution but failing homogeneity of variance, the Welsh test was conducted. The Mann-Whitney U test for non-normally distributed variables was used. The dichotomous data were analyzed by the chi-square test or chi-square with Yates' correction. Variables statistically significantly in univariate analysis (significance level $p < 0.05$) were used for the construction of a multivariate logistic regression model. The quality of the models and the usefulness of the markers were evaluated using receiver operating characteristic (ROC) curves and tables of reclassification.

For quantitative variables (continuous and discrete) to evaluate correlations between variables, Spearman's rank correlation coefficient was used.

## 3. Results

### 3.1. General characteristics of patients

Out of 520 ischemic stroke patients, 65 patients were assigned to the cryptogenic stroke (CS) group and compared to 36 patients without stroke. CS was defined using TOAST (Trial of Org 10,172 in Acute Stroke Treatment) criteria as brain infarction that is not attributable to a source of definite cardioembolism, large artery atherosclerosis, or small artery disease despite a standard vascular, cardiac, and serologic evaluation. Basic characteristics of patients are presented in Tables 1 and 2.

CS group patients had from 1 to 15 points according to the National Institutes of Health Stroke Scale (NIHSS), mean 5.07 points, so they were patients with minor and moderate stroke severity.

There were no differences in blood pressure or body mass index (BMI) values between the groups. Among the patients with CS 52% had stable hypertension, 9% had coronary artery disease, and 38% were smokers. In the control group 43% of patients had hypertension, 9% had coronary artery disease and 13% were smokers. Eight percent of strokes were complicated by a hemorrhage to the central nervous system. There were no statistically significant differences in the treatment between the groups. Patients in the CS group had a higher level of GFR [75 mL/min/1.73 $m^3$ (64–89) vs 68 (62–78); $p = 0.002$] compared to the control group. There were no differences between groups in activated partial thromboplastin time (APTT) or prothrombin time (PT). There was no statistically significant difference in the cIMT between the groups.

The level of left ventricular ejection fraction (LVEF) was statistically significantly higher in patients without stroke compared to patients with CS [60% (55–64) vs 63% (60–66) $p = 0.009$]. The LVEF values were in the normal range in both groups. Compared to the control

**Table 1**
Basic characteristics of patients in both groups.

| Parameter | Patients with cryptogenic stroke | Controls | p |
|---|---|---|---|
| **Number of patients** | 65 | 36 | 0.89 |
| **Median age (years)** | 54 (47–58) | 53 (47–58) | 0.001 |
| **Gender (male) (%)** | 42 | 61 | 0.059 |
| **BMI (kg/ m$^{2)}$** | 26 (22.4–28.7) | 25 (21.8–28.1) | 0.49 |
| **BSA (m$^2$)** | 1.87 (1.75–1.99) | 1.9 (1.71–2.05) | 0.73 |
| **Hypertension (%)** | 52 | 43 | 0.36 |
| **CAD (%)** | 9 | 9 | 0.76 |
| **Smoking (%)** | 38 | 13 | 0.02 |
| **Hemorrhagic CNS (%)** | 8 | – | – |
| **ASA (%)** | 23 | 9 | 0.12 |
| **Statin (%)** | 23 | 17 | 0.66 |
| **Beta blocker (%)** | 28 | 12 | 0.13 |
| **ACE (%)** | 37 | 24 | 0.2 |
| **CCB (%)** | 20 | 9 | 0.27 |
| **Insulin (%)** | 8 | 0 | 0.23 |
| **Diuretic (%)** | 20 | 15 | 0.75 |
| **cIMT (mm)** | 0.8 (0.7–1.15) | 0.8 (0.7–0.9) | 0.42 |
| **SBP (mmHg)** | 136 (±18.7) | 128 (±19.7) | 0.053 |
| **DBP (mmHg)** | 83 (±9.9) | 82 (±9.0) | 0.72 |

ASA - acidum acetysalicylicum; ACE - angiotensin-converting enzyme; BMI - body mass index; BSA - body surface area; CAD - coronary artery disease; CCB - calcium-channel blocker; DBP - diastolic blood pressure; cIMT - carotid intima media thickness; CNS - central nervous system; PFO - persistent foramen ovale; SBP - systolic blood pressure.

**Table 2**
Evaluation of basic biochemical parameters in both groups.

| Parameter | Patients with cryptogenic stroke | Controls | p |
|---|---|---|---|
| **$K^+$ (mmol/L)** | 4.08 (±0.35) | 4.21 (±0.28) | 0.058 |
| **$Cl^-$ (mmol/L)** | 103.81 (±4.0) | 104.80 (±2.4) | 0.21 |
| **$Na^+$ (mmol/L)** | 139.2* (137.4-140.8) | 139.2*(138.2-140.0) | 0.63 |
| **GFR (mL/min/ 1.73 m$^3$)** | 75* (64-89) | 68 (62-78) | 0.0002 |
| **Creatinine (µm/l)** | 1.58* (1.11-2.0) | 1.3* (0.86-1.7) | 0.21 |
| **APTT (ratio)** | 28.1* (25.9-31.3) | 28.7* (26.5-31.7) | 0.42 |
| **PT (sec)** | 0.9* (0.8-1.0) | 0.9* (0.8-0.95) | 0.94 |
| **APPT (sec)** | 89.7* (77.1-102) | 106.1* (95.9-110.5) | 0.42 |

APTT - activated Partial Thromboplastin Time; $Cl^-$ - chlorine; GFR - glomerular filtration rate; $K^+$ - potassium; $Na^+$ - sodium; PT - prothrombin time.
* - median.

group, CS patients had a statistically significantly higher LV mass index (LVMI) (p = 0.0004), increased E/E' ratio (p = 0.0002) and increased left atrial (LA) diameter (p < 0.0001).

Mean left atrial volume index ml/m$^2$ (LAVI) was also higher in patients with CS (p = 0.002). The tricuspid annular systolic excursion (TAPSE) and left ventricle (LV) diameters were similar between the groups. The evaluation of basic echocardiographic parameters among the groups is presented in Table 3.

### 3.2. Biomarker analysis

There were no differences between the CS and control groups in the levels of syndecan 4, resistin, leptin, PIIINP, LDL cholesterol, or triglycerides. The level of HDL cholesterol was statistically significantly lower in the CS group than in controls [1.1 mmol/L (0.95–1.46) vs 1.37 (1.19–1.6); p = 0.02]. Patients in the stroke group had higher levels of NT-proBNP [391 pg/ml (107–1249) vs 109 (46–236); p = 0.003] (Fig. 1), IL6 [2.6 pg/ml (0.8–8.1) vs 0.7 (0.4–1.2); p = 0.002] (Fig. 2) and ADMA [0.44 µmol/L (0.39–.55) vs 0.36 (0.32–0.4); p = 0.0002] (Fig. 3) than the control group. The evaluation of selected biomarker levels is presented in Table 4. In the analysis of biomarkers and their

**Table 3**
Evaluation of echocardiographic parameters in both groups.

| Parameter | Patients with cryptogenic stroke | Controls | p |
|---|---|---|---|
| **LVEF %** | 60 (55–64)* | 63 (60–66)* | 0.0098 |
| **E/E'(cm/s)** | 7.61 (6.1–8.99)* | 6.07 (5.32–6.96)* | 0.0002 |
| **LVMI (g/m$^2$)** | 112 (90–125)* | 89.5 (77–101)* | 0.0004 |
| **LAVI (ml/m$^2$)** | 27.40 ± 11.3 | 20.91 ± 6.6 | 0.002 |
| **LA (mm)** | 36 (33–41)* | 35 (32–38)* | 0.07 |
| **TAPSE (mm)** | 24 (21–27)* | 25 (22–28)* | 0.34 |
| **LV diastole (mm)** | 43 (40–46)* | 45 (42–48)* | 0.17 |
| **LV systole (mm)** | 32 (27–36)* | 29 (27.0–33)* | 0.10 |

For the parameters with non-normal distribution there are given median values (lower and higher values). For the parameters with normal distribution there are given mean values ± standard deviation (SD). [1] p t Student. [2] p Levenea. E/E' - ratio of peak velocity of early diastolic transmittal flow to peak velocity of early diastolic mitral annular motion as determined by pulsed wave Doppler; LA - left atrium; LAVI - left atrial volume index; LV - left ventricle; LVEF - left ventricular ejection fraction; LVMI - left ventricular mass index; TAPSE - tricuspid annular plane systolic excursion.
* - median.

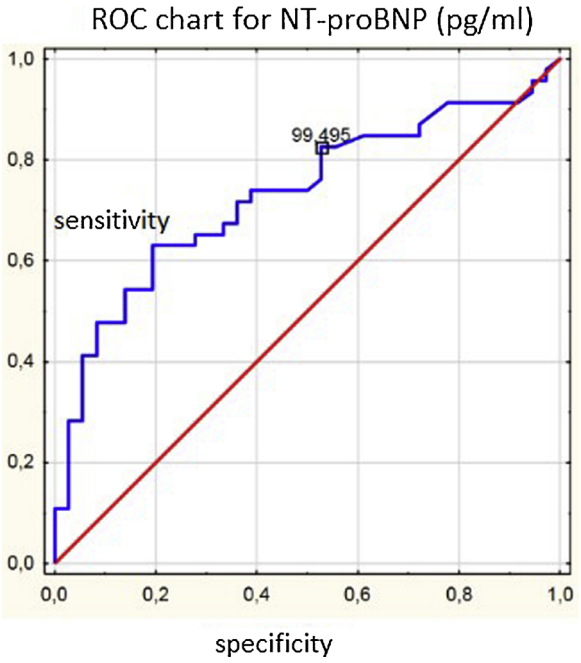


**Fig. 1.** ROC chart for NT-proBNP.

correlations with $CHA_2DS_2$-VASc and NIHSS, we observed a statistically significant positive correlation between ADMA level and points in both scales and also a positive correlation between the level of IL-6 and points in the NIHSS scale – Table 5.

### 3.3. Multivariate logistic regression analysis

In the multivariate analysis the only biomarker statistically significantly associated with the occurrence of CS was IL-6 (odds ratio [OR] 1.918, 95% confidence interval [CI] 1.029–3.575; p = 0.04) (Table 6).

## 4. Discussion

### 4.1. Brief description of results

The results of our study show that the levels of ADMA, NT-proBNP and IL-6 are higher in CS patients. The increase in the inflammatory

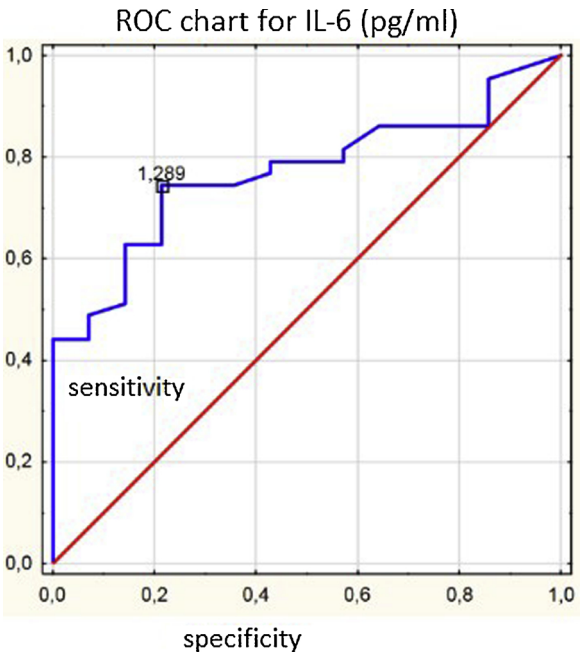


**Fig. 2.** ROC chart for IL-6.

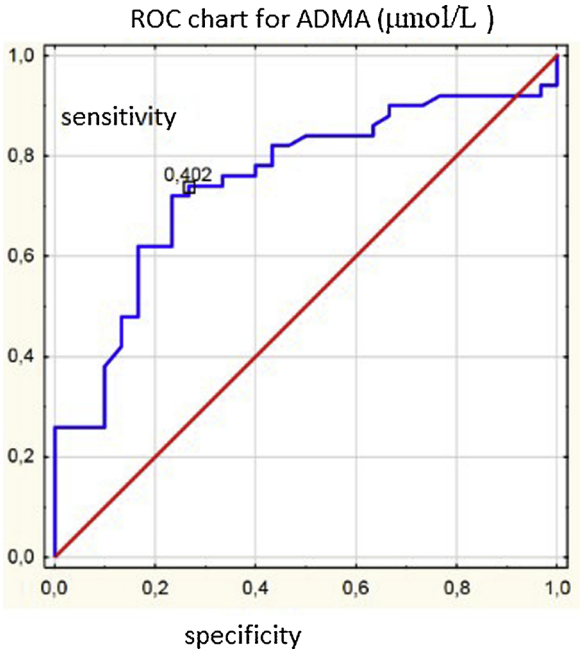


**Fig. 3.** ROC chart for ADMA.

cytokine IL6 by 1 pg/ml increased the risk of CS almost two-fold. We concluded that the endothelial dysfunction and inflammation may have synergic effects on cardiovascular risk and CS occurrence. The biomarkers reflecting endothelial dysfunction - e.g. ADMA, and inflammation, - e.g. IL-6, correlate with clinical state of CS patients assessed with the NIHSS scale [18].

In the pathogenesis of stroke, genetic disorders such as Anderson-Fabry disease may also have an important role. According to Tuttolomondo et al. [19] it may cause about 4% of CS in young patients.

The main involvement of the central nervous system is attributable to cerebral vasculopathy, with an increased incidence of stroke [20]. Because of known connections between some genetic disorders and strokes, in our study all patients with CS had a genetic assessment before enrollment in the study.

### 4.2. ADMA and its significance in stroke

The experience of recent years indicates that expansion of tissues damage after stroke is the result of local microcirculation deterioration and destruction mechanisms connected with inflammation and the immunological response to ischemia. The process of monocyte adhesion to endothelial cells induced by ADMA represents a decisive mechanism for the initiation of atherosclerosis. Experimental studies suggested that ADMA is also involved in further steps of atherosclerosis, such as induction of vascular smooth muscle cell (VSMC) migration, foam cell formation and apoptosis of VMSCs and endothelial cells.

By inhibiting the formation of endothelial NO, ADMA accumulation may result in endothelial dysfunction in the LA and the LA appendage, which may lead to thrombus formation and subsequent thromboembolic events. The decrease in NO availability could enhance atherosclerosis and local inflammation of the vessel wall, which may play an important role in plaque rupture, thereby leading to stroke. The infusion of ADMA could increase arterial stiffness and decrease cerebral perfusion [22]. ADMA might be the link between increased oxidative stress, endothelial dysfunction, thrombogenesis and atherosclerosis, and could therefore represent a risk factor of stroke [21]. In the study by Böger [22], based on 225 hemodialyzed patients observed during 34 months, the level of ADMA and age were the strongest predictors of future cardiovascular events and mortality. It remains an open issue whether ADMA is only a marker of atherosclerosis incidents or is also an active substance taking part in them.

Solovyeva et al. [23] revealed that the serum levels of ADMA and high-sensitivity CRP (hsCRP) statistically significantly increase in the acute period of atherothrombotic stroke. An elevated ADMA level was statistically significantly associated with an increased predicted stroke risk (10-year risk, ≥ 5%), suggesting that measuring the ADMA level is useful for identifying Japanese women and men with an elevated stroke risk [24]. In the study of Lao et al. [25] ADMA correlates with stroke risk according to the $CHADS_2/CHA_2DS_2$-VASc score. Also in our study, ADMA positively correlated with $CHA_2DS_2$-VASc score, which suggests that ADMA may become a new biomarker for predicting pro-thrombotic risk. Among healthy individuals with a low risk of cardiovascular disease according to the Systematic Coronary Risk Evaluation (SCORE) model, those with plasma ADMA levels above 0.71 µmol/L had a higher risk of cardiovascular and cerebrovascular events, as compared to those with plasma ADMA levels below this value [26,27]. A prospective study in 125 patients with type 2 diabetes demonstrated a statistically significant association of elevated plasma levels of ADMA at baseline with large vessel cardiovascular events and stroke after a 21-month follow-up [28]. ADMA > 0.63 µmol/L was predictive of incident cardiovascular events compared with those with ADMA ≤ 0.53 µmol/L (2.37 [95% CI 1.05–5.35]; p = 0.038) [28]. In our study the value of ADMA that increases the risk of CS stroke is >0.4 µmol/L ADMA concentration might also suggest the type of stroke. In a Swedish study [29], in 363 stroke patients, the serum concentrations of ADMA were increased in the subgroups with TIA or cardioembolic stroke, but not in non-cardioembolic stroke and hemorrhagic stroke. A contradictory result was found in a study with 238 Hispanic patients [30] where plasma ADMA concentrations after acute stroke did not differ from those in healthy controls. The authors indicated that differences between the studies might be due to different genetic, socioeconomic and nutritional factors [31]. In our study ADMA level was statistically significantly higher in patients with cryptogenic stroke compared to healthy controls

### 4.3. NT-proBNP and its significance in stroke

In the study of Satoh et al. [26] NT-proBNP was statistically significantly associated with an elevated stroke risk. With the maximum follow-up period set at 5 years, the hazard ratio for stroke of the NT-

**Table 4**
Evaluation of biomarkers in both groups.

| Parameter | Patients with cryptogenic stroke (median) | Controls (median) | P |
|---|---|---|---|
| **Syndecan 4 (ng/ml)** | 0.81 (0.78 - 0.87) | 0.79 (0.78-0.88) | 0.68 |
| **Adipokines:** | | | |
| **- leptin (ng/ml)** | 23.96 (7.83-51.5) | 30.27 (13.9-81.89) | 0.24 |
| **- adiponectin (µg/ml)** | 6.65 (3.99-10.03) | 6.62 (4.47-8.80) | 0.70 |
| **- resistin (ng/ml)** | 4.51 (3.14-7.4) | 4.12 (3.04-6.24) | 0.38 |
| **LDL cholesterol (mmol/l)** | 2.83 (2.07-4.0) | 3.02 (2.66-3.67) | 0.37 |
| **Triglycerides (mmol/l)** | 1.6 (1.13-2.02) | 1.36 (0.86-1.76) | 0.257 |
| **HDL cholesterol (mmol/l)** | 1.19 (0.95-1.46) | 1.38 (1.21-1.6) | 0.011 |
| **NT- pro BNP (pg/ml)** | 391.13 (107.9-1249.22) | 109.31 (46.22-236.90) | 0.0003 |
| **IL- 6 (pg/ml)** | 2.7 (0.89-8.14) | 0.69 (0.43-1.22) | 0.002 |
| **ADMA (µmol/l)** | 0.45 (0.39-.056) | 0.37 (0.33-0.40) | 0.0002 |
| **PIIINP (ng/ml)** | 16.44(14.11 -21.07) | 14.96 (12.97-16.30) | 0.06 |

ADMA - asymmetric dimethylarginine; cIMT - carotid intima-media; HDL - high density lipoprotein; IL-6 - interleukin 6; LDL - low density lipoprotein; NT-proBNP - N-terminal pro-brain natriuretic peptide; PIIINP - N-terminal propeptide of type III procollagen.

**Table 5**
Significant correlations between biochemical parameters and CHA2DS2VASc and NIHSS scales.

| Parameter | R Spearman | p |
|---|---|---|
| **ADMA and CHA2DS2VASc scale** | 0.36 | 0.001 |
| **ADMA and NIHSS scale** | 0.46 | 0.000016 |
| **IL-6 and NIHSS scale** | 0.55 | 0.000009 |

ADMA - asymmetric dimethylarginine; CHA2DS2VASc - congestive heart failure, hypertension, age ≥75 years, diabetes mellitus, stroke or Transient Ischemic attack or thromboembolism, vascular disease, age 65–74 years, sex category; Il-6 - interleukin 6; NIHSS - National Institutes of Health Stroke Scale.

**Table 6**
The multivariate analysis - stepwise logistic regression.

| Variable | OR | 95% CI for OR | | p-value |
|---|---|---|---|---|
| | | Lower limit | Upper limit | |
| IL-6 | 1.9 | 1.02 | 3.5 | 0.04 |

Il-6 - interleukin 6; OR - odds ratio.

proBNP ≥ 125.0-pg/mL group compared with the < 30.0-pg/mL group increased statistically significantly (HR, 4.51; 95% CI: 1.03–19.85). BNP and NT-proBNP display closely equivalent overall diagnostic accuracies in distinguishing cardioembolic stroke from non-cardioembolic stroke in adult ischemic stroke patients [32]. Increased blood levels of natriuretic peptides (BNP/NT-proBNP) have been repeatedly associated with cardioembolic stroke. Llombart et al. [33] collected information on 2834 patients with a defined cause of stroke. BNP/NT-proBNP levels were statistically significantly elevated in cardioembolic stroke until 72 h from symptoms onset. Predictive models showed a sensitivity > 90% and specificity > 80% when BNP/NT-proBNP were added considering the lowest and the highest quartile, respectively. We found that the values of NT-proBNP are higher in patients with CS compared to controls, which suggests that in part CS can have underlying cardioembolic etiology. The value of NT-proBNP which statistically significantly increases the risk of stroke in our present study (> 99 pg/ml) was lower than the value that we use to diagnose heart failure.

### *4.4. IL-6, inflammation and their significance in stroke*

IL-6 has some pro-inflammatory effects which may be essential to the induction and evolution of early inflammatory injury in the brain and its vasculature. IL6 has been shown to exert pro-inflammatory action on brain microvascular endothelial cells [32]. IL-6 released after stroke impairs cerebrovascular autoregulation and increases histopathology [31]. Generally, elevated IL-6 expression is found in patients with acute ischemic stroke and is also reported to be accompanied by decreased cognitive functioning [34]. The results of Yang et al. [35] suggest that polymorphisms in IL-6-174G > C and -572C > G are associated with ischemic stroke risk in young patients, and that these polymorphisms interact with hypertension, obesity and etiologic subtypes. Moreover, Madoka et al. [36] reported that the magnitude of the increase in IL-6 and CRP from day 0 to day 2 was correlated with the size of brain infarction, and the increase in IL-6 and CRP during hospitalization influenced the poor outcome at discharge. Interleukin-6 in our present study was independently associated with CS. The value of IL-6 which statistically significantly increases the risk of CS in our study is >1.2 pg/ml. Therefore, we hypothesized that IL-6 might be involved in the pathophysiologic process of stroke. In the study of Shin Na et al. [37] CS subjects had statistically significantly higher E-selectin ($p = 0.046$), IL-6 ($p = 0.040$), and hs-CRP ($p = 0.001$) compared with controls. Similarly, for ischemic strokes, IL-6 showed the strongest univariate association (> two-fold increase) in a large sample study [38]. Inflammation, which is in our study represented by a higher level of IL-6, may have a possible role in the development of atrial cardiopathy, currently considered a potential pathogenic factor underlying CS even independently from the occurrence of atrial fibrillation together with the autonomic nervous system (ANS), which plays a well-known role in determining statistically significant and heterogeneous electrophysiological changes of atrial cardiomyocytes [39]. Systemic inflammation may play a role in a fraction of patients with CS by increasing AF risk via atrial electric remodeling [40] and also with the occurrence of paroxysmal silent AF [41].

### *4.5. Limitations of the study*

After patients' hospital admissions with the diagnosis of ischemic stroke, we had to eliminate basic causes of stroke using echocardiography, Holter ECG and blood tests, which took about 7 days. After that time, we could take blood to assess the levels of selected biomarkers. We are not able to exclude the possibility that the levels of biomarkers are the consequence of ischemic stroke instead of the cause of the stroke. The study also involved a relatively small number of patients and the findings need to be confirmed in a larger population.

## 5. Conclusion

Nitric oxide is a known regulator of vascular endothelial function. Elevated levels of pro-inflammatory markers may interfere with signaling and reduce NO release, which would result in decreased vascular endothelial function [42]. Pro-inflammatory markers such as IL-6 and ADMA which interfere with NO bioavailability may have a negative effect on peripheral vascular endothelial function starting in the acute stroke setting.

In our study endothelial dysfunction assessed by increased level of ADMA affects the inflammatory state in patients with cryptogenic stroke. Increase in the inflammatory cytokine IL-6 by 1 pg/ml almost doubles the risk of stroke. Biomarkers reflecting endothelial dysfunction, e.g. ADMA, and inflammation, e.g. IL-6, correlate with the clinical state of CS patients assessed with the NIHSS scale. Also, ADMA level correlates positively with $CHA_2DS_2$-VASc score. ADMA, IL-6 and NT-proBNP can be recommended for inclusion in the clinical molecular panel for personalized diagnostics of causes of stroke along with clinical anamnestic data.

To sum up, ADMA and inflammatory factors, especially IL-6, might be biochemical predictors of stroke, along with already known risk factors. ADMA and IL-6 may be markers of stroke outcome.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Financial disclosure

The study was financed by research grants no. 502-03/5-139-02/502-54-203 and 502-03/5-139-02/502-54-229-18 of the Medical University of Lodz, Poland.

## The author contribution

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Data interpretation: Paulina Gąsiorek, Agata Bielecka-Dąbrowa, Maciej Banach
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