The importance of selected markers of inflammation and blood-brain barrier damage for short-term post-stroke prognosis

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Background

Stroke causes an immediate local immuno-inflammatory reaction. It is characterized by a strong activation of microglia, astrocytes and vascular endothelial cells; an inflow of inflammatory cells (primarily neutrophils, then macrophages and monocytes); an activation of adhesive molecules and the release of cytokines both from activated cells and the endothelium. Following brain ischemia, a non-specific response is triggered, resulting in the elimination of dead cells from the damage zone and in the protection of surviving neurons from excitotoxicity. In a later stage, specific response mechanisms are activated; these are significant for the regeneration and neuroplasticity [1].

The mechanism of post-stroke immune activation is poorly understood. Studies on experimental stroke suggest that cytokines modulate the process of neuronal damage during ischemic stroke. Although TNF and interleukin 1, 2 and 6 are the most frequently studied cytokines in stroke patients, the conclusions are ambiguous and inconsistent with the results of experimental stroke studies [2-4]. Increased blood concentrations of NSE and/or S100B after stroke indicate that the BBB has been damaged; they are associated with a worse neurological status. Still little is known about the importance of progranulin and other markers of post-stroke prognosis.

The aims of this project were: to assess the blood serum concentration of the selected markers of inflammation, of the BBB and nervous tissue damage, and of the coagulation system; we also wanted to assess the markers of neuro and angiogenesis in patients on the first day of ischemic stroke, and the mutual correlations between these marker levels. An additional aim was to assess the importance of the above parameters for the patient's neurological state on the first day, and their functional status on the 30th day of stroke.

Methods

Our prospective study conducted in 2016-2017 included patients with first-in-life stroke, as manifested clinically and identified according to WHO clinical criteria, who had an acute ischemic lesion of the brain visualized in computed tomography and/or magnetic resonance imaging of the head. The other main inclusion criteria were: the length of time from the onset of stroke symptoms to hospital admission ≤ 24 hours, and pre-stroke status according to modified Rankin Scale (mRS) ≤ 1 point.

All patients included in the study were analyzed according to:

- their age at first-in-life stroke;
- the presence of comorbidities, such as atrial fibrillation (AF), arterial hypertension (AH), coronary heart disease (CHD), diabetes mellitus (DM), lipid disorders (LD), >70% atherosclerotic carotid artery stenosis (CAS, ipsilaterally to the acute ischemic brain lesion);
- their neurological status on the first day of stroke, evaluated on the NIHSS (National Institute of Health Stroke Scale); [5]
- plasma concentration of the following markers on the first day of stroke: interleukins IL-2 and IL-6; calcium-binding protein B, tumor necrosis factor alfa, progranulin, neuron-specific enolase, urokinase-type plasminogen activator (uPA), vascular endothelial growth factor (VEGF); brain-derived neurotrophic factor (BDNF), C reactive protein (CRP), leucocyte and platelet (PLT) counts;
- their functional status at 30 days following stroke as per the mRS scale. [6]

The diagnosis of AH was consistent with the recommendations of the European Society of Cardiology (ESC); DM was diagnosed according to the criteria of the Diabetes Association; dyslipidemia was defined according to the ESC recommendations (Guidelines for the Management of Dyslipidemias). [7-9]

The degrees of stenosis of the common carotid artery and/or internal carotid artery were assessed according to the NASCET criteria. [10]

Blood BDNF, IL-2, IL-6, S100B, TNF, GRN, NSE, uPA, VEGF concentrations on day 1 of stroke were assessed according to procedure as follows: 7 ml of EDTA blood was drawn and plasma was obtained after 15 minutes of centrifugation (1500 rpm), then the plasma was frozen at -80°C. The concentrations of above listed markers were measured using the ELISA method (Magnetic Luminex Assay R&D Systems). The assessments were performed at the Department of Physiology, Medical University of Silesia in Katowice.

Patients were categorized into three subgroups based on the NIHSS score ($A \leq 4$, $B 5-12$ and $C > 12$). In each of these subgroups the mean concentrations of the examined substances were assessed and comparisons were made between them

Patients were categorized into three subgroups based on the mRS score ($D \leq 2$ and $E = 3$, $F > 3$). In each of these groups formed the mean concentrations of the examined substances were assessed and comparisons were made between those subgroups.

A subgroup was formed of patients with symptomatic atherosclerosis of the coronary arteries (CHD and/or MI) and/or carotid arteries (>70% stenosis) / cerebral arteries (>50%) and ones with atherogenic stroke/TIA in the last 6 months, for which a profile of test concentrations was assessed and compared with the parameters in other patients.

A subgroup was formed of ‘the best clinical benefits’ (BB) patients; it consisted of those with scores >10 on the NIHSS scale (the first day) who scored ≤ 2 on the mRS scale on the 30th day following the onset of stroke. For that subgroup, the profile of test concentrations was assessed and compared with the parameters in other patients.

Univariate analyses for differences between male and female groups for binary variables AH, DM, AF, LD, CHD, CAS, AT, therapy were performed using the Pearson's Chi-squared test. Continuous variables were compared between male and female patients using Welch's ANOVA or Kruskal-Wallis testing, as appropriate. We also analysed NIHSS and mRS grouped into 3-category ordinal scale. In this case for all not censored variables including WBC, PLT, CRP and cytokines we use a one-way ANOVA or a Kruskal-Wallis test. For censored data with values below the detection limit (LOD) we use the maximum likelihood estimation procedure or a Peto test accordingly. For all significant test results we performed post-hoc tests on pair-wise comparisons. The variable correlation plot was determined using the pearson correlation coefficient.

Multivariable models were built by using logistic regression and binomial GLM for dichotomous outcomes (eg, best benefits group) and ordinal logistic regression for ordinal outcomes (NIHSS, mRS). The model variable selection procedures included automatic selection (stepwise, forward and backward) based on AIC- and BIC criterion. For evaluating the accuracy of the model predictions the “leave one out” procedure and the area under the ROC curves (AUC) or multiclass AUC estimator was used. All statistical analyses were performed using R version 3.4.2.

The study was accepted by the Ethics Committee of the Silesian Medical University of Silesia in Katowice.

Results

The study included 138 patients on the first day of ischemic stroke hospitalized in the Department of Neurology, Medical University of Silesia in Katowice: 75 patients were women,

which accounted for 53.96% of all subjects. Mean age of the study subjects: 73.11 ± 11.48 [36-103]. The mean age of women was significantly higher; the neurological status on the first day of stroke and the functional status on the 30th day were significantly more severe than those in men. Mean PLT count and the incidence of DM were significantly higher among women. In the comparative analysis, the differences between other parameters during inclusion were not statistically significant.

The clinical characteristic of the patients is presented in Table 1 (Tab. 1).

Table 1. Characteristics of the study participants

Parameter	All	Female	Male	p-value
Age (years), mean $\pm$ SD [min, max]	73.11 $\pm$ 11.48 [36-103]	75.42 $\pm$ 10.92 [43-103]	70.40 $\pm$ 11.71 [36-92]	0.0101*
NIHSS, median (IQR) [min, max]	3 (6) [1-18]	5 (8) [0-18]	2 (3.5) [0-15]	0.0007***
mRS, median (IQR) [min, max]	2 (3) [0-6]	3 (3) [0-6]	1 (2) [0-5]	0.0314*
ST, n, (%) (A/S/C/O/D)	19/45/40/33/2 (14/32/29/24/1)	10/29/20/15/1 (13/39/27/20/1)	9/16/20/18/1 (14/25/31/28/2)	0.5582
WBC, mean $\pm$ SD [min, max]	8.52 $\pm$ 3.09 [3.02-18.94]	8.53 $\pm$ 3.06 [3.43-17.32]	8.50 $\pm$ 3.15 [3.02-18.94]	0.9545
PLT, mean $\pm$ SD [min, max]	233.67 $\pm$ 69.11 [76-542]	255.12 $\pm$ 65.05 [121-542]	211.71 $\pm$ 67.84 [76-435]	0.0005***
CRP, median (IQR) [min, max]	5 (17.92) [0.5-218]	5.05 (17.4) [0.5-160]	5 (19.17) [5-218]	0.9389
AH, n (%)	127 (91)	68 (91)	59 (92)	0.7681
DM, n (%)	49 (35)	26 (35)	23 (36)	0.0244*
AF, n (%)	44 (32)	23 (31)	21 (33)	0.8695
LD, n (%)	62 (45)	23 (31)	21 (33)	0.2548
CHD, n (%)	71 (51)	38 (51)	33 (52)	1
CAS, n (%)	14 (10)	7 (9)	7 (11)	1
HT, n (%)	5 (4)	3 (4)	2 (3)	1
Therapy, n (rtPA#/APT only)	33/106	20/55	13/51	0.3968

NIHSS- National Institutes of Health Stroke Scale, mRS- modified Rankin Scale, ST- stroke phenotyping (A- atherosclerosis, S- small vessel disease, C- cor, O- other, D- dissection), WBC- white blood count, PLT- platelet, CRP- C reactive protein, AH- arterial hypertension, DM- diabetes mellitus, AF- atrial fibrillation, LD- lipid disorders, CHD-coronary heart disease, CAS- carotid artery stenosis, HT- Hemorrhagic transformation, rtPA- recombinant tissue plasminogen activator, APT- antiplatelet

*P < 0.05; **P < 0.01; ***P < 0.001

including middle cerebral artery thrombectomy in 7 patients

Differences between male and female groups for binary variables AH, DM, AF, LD, CAD, CAS, HT, therapy were analyzed using the Pearson's Chi-squared test. Continuous variables were compared between male and female using Welch's ANOVA or Kruskal-Wallis testing, as appropriate.

Patients with a higher score on the NIHSS than those obtaining lower scores showed significantly higher concentrations of TNF-alpha, WBC, CRP, NSE, IL-6 and S100B. (Tab. 2)

The differences between other parameters were not statistically significant.

Table 2. The comparison of selected parameters between NIHSS and mRS subgroups.

NIHSS						
Parameter	Test	p-value	FDR corrected p-value	Post-hoc test p-value B-A	Post-hoc test p-value C-A	Post-hoc test p-value C-B
WBC	Anova	0.00496	0.01289	0.01596	0.05830	0.28422
CRP	Kruskal	0.00083	0.00361	0.00600	0.00926	0.10253
NSE	Kruskal	0.02019	0.04374	0.02093	0.37730	0.84634
IL-6	MLE	0.00001	0.00006	0.00116	0.00023	0.00740
S100B	MLE	<0.00001	<0.00001	<0.00001	0.00028	0.05232
TNFalfa	Peto	0.00218	0.00709	0.42851	0.00864	0.03518

mRankin						
Parameter	Test	p-value	FDR corrected p-value	Post-hoc test p-value E-D	Post-hoc test p-value F-D	Post-hoc test p-value F-E
WBC	ANOVA	<0.00001	0.00002	0.00824	<0.00001	0.00627
CRP	Kruskal	<0.00001	0.00002	0.00054	<0.00001	0.02640
GRN	ANOVA	0.01411	0.03669	0.56047	0.01129	0.02865
IL-6	MLE.test	0.00002	0.00007	0.00925	0.00003	0.02894
S100B	Peto	0.00002	0.00002	0.02357	0.00006	0.01736

WBC- white blood counts, CRP- C reactive protein, NSE- neuron-specific enolase, IL-6- interleukin 6, S100B- calcium- binding protein B, TNF α - tumor necrosis factor α

A $\leq$ 4 points according to NIHSS, B 5-12, C $>$ 12

D $\leq$ 2 points according to mRS, E = 3, F $>$ 3

Patients with a higher score on the mRS than those obtaining lower scores showed significantly higher concentrations of WBC, CRP, GRN, IL-6, S100B. (Tab. 2) The differences between other parameters were not statistically significant.

Factors with an independent influence on the neurological status on the first day of stroke were: sex, WBC, PLT, CRP, S100B and IL-6 levels. (Tab. 3)

Table 3. Regression analysis of the influence of clinical phenodata and cytokines on NIHSS

Coefficients	OR	CI 95%	P-value
sex	0.019	(0-0.25)	0.038
age	0.935	(0.831-0.998)	0.099
DM	0.082	(0.002-0.715)	0.062
LD	0.162	(0.007-0.889)	0.107
AH	0	(0-0.015)	0.064
WBC	46.037	(3.684-7507.14)	0.02
PLT	1753.457	(729.714-6914.231)	0.019
CRP	8.36	(2.449-107.925)	0.013
IL-2	3.235	(0.993-22.601)	0.094
IL-6	4.378	(1.785-32.064)	0.02
S100B	5.338	(2.038-35.645)	0.01
VEGF_C	0	(0-0.106)	0.097

DM- diabetes mellitus LD- lipid disorders AH- arterial hypertension, WBC- white blood count, PLT- platelet, CRP- C reactive protein, IL-6- interleukin 6, IL-2- interleukin 2, S100B- calcium-binding protein B, VEGF-C- vascular endothelial growth factor C

Atrial fibrillation, leukocyte count, CRP, NSA, uPA, interleukin 6 and S100B showed an independent impact on the functional status on the 30th day of stroke. (Tab. 4)

Table 4. The regression analysis of the influence of clinical phenodata without gender feature and cytokines on mRS

Coefficients	OR	CI 95%	P-value
AF	6.162	(1.981-30.917)	0.006
CHD	2.75	(1.065-9.474)	0.056
CAS	0.114	(0.009-0.741)	0.044
WBC	23.158	(3.269-365.421)	0.005
PLT	6.22	(1.166-57.467)	0.054
CRP	2.583	(1.593-5.419)	0.001
NSE	0.247	(0.072-0.573)	0.004
uPA	0.102	(0.008-0.694)	0.031
IL-6	1.512	(1.069-2.249)	0.022
S100B	3.286	(1.757-8.013)	0.001

mRS- modified Rankin Scale, AF- atrial fibrillation, DM- diabetes mellitus CAS- carotid artery stenosis, WBC- white blood count, PLT- platelet, CRP- C reactive protein, NSE- neuron-specific enolase, uPA- urokinase-type plasminogen activator, IL-6- interleukin 6, S100B- calcium-binding protein B, VEGF-C- vascular endothelial growth factor C

Patients who reached a good functional status on the 30th day, despite an initial poor neurological status, as compared to others, had significantly higher TNF alpha, NSE, IL-2 and VEGF levels. (Tab. 5)

Table 5. Logistic regression analysis of the influence of cytokines into the neurological improvement in patients with the best clinical benefits.

Coefficients	OR	CI 95%	P-value
BDNF	22.809	(1.451-222.998)	0.098
TNFalfa	9.865	(9.777-10.145)	0.04
VEGF	0.695	(0.685-0.898)	0.054
NSE	3049.504	(13.636-1.58E+04)	0.044
IL-2	0.001	(0.001-0.123)	0.044
S100B	0.002	(0.001-0.002)	0.057
VEGF_C	4.48E+05	(83.673-1.56E+06)	0.036

BDNF- brain-derived neurotrophic factor, TNFalfa- tumor necrosis factor alfa NSE- neuron-specific enolase, IL-2- interleukin 2, S100B- calcium-binding protein B, VEGF-C- vascular endothelial growth factor C

Patients with symptomatic atherosclerosis of carotid/cerebral and/or coronary arteries, as compared to others, were older ($p=0.003$) and had higher levels of CRP, IL-6, and S100B. In each case, the differences were statistically significant (Tab. 6).

Table 6. Logistic regression analysis of the importance of cytokines in patients with symptomatic atherosclerosis.

Coefficients	OR	CI 95%	P-value
Age			0.003
BNDF	0.705	(0.365-1.062)	0.207
WBC	3.215	(1.043-13.254)	0.068
CRP	0.603	(0.346-0.91)	0.025
uPA	3.653	(0.904-24.63)	0.124
IL-6	0.593	(0.324-0.88)	0.017
S100B	1.646	(1.044-3.158)	0.036
VEGF_C	0.207	(0.01-1.16)	0.106

BDNF- brain-derived neurotrophic factor, WBC- white blood count, CRP- C reactive protein, uPA- urokinase-type plasminogen activator, IL-6- interleukin 6, S100B- calcium-binding protein B, VEGF-C- vascular endothelial growth factor C

There were clear correlations between the following factors: a positive correlation for the NSE and S100B pair, and for the BDNF and VEGF, as shown in Figure 1. (Fig. 1)

Figure 1. Correlation plot of all analysed features.

BDNF- brain-derived neurotrophic factor, TNFalfa- tumor necrosis factor alfa, VEGF-C- vascular endothelial growth factor C, WBC- white blood count, PLT- platelet, CRP- C reactive protein, NSE- neuron-specific enolase, GRN- progranulin, uPA- urokinase-type plasminogen activator, IL-2- interleukin 2, IL-6- interleukin 6, S100B- calcium-binding protein B

Discussion

Acute cerebral ischemia triggers local and systemic immune response. Immediately after the ischemic event, microglia, mast cells, and astrocytes are activated. These form the initial source of cytokines which cause the BBB permeability and encourage the migration of inflammatory cells from the periphery into the brain. [11] During the first few hours, neutrophils penetrate

into the hypoxemic tissue which is invaded by macrophages and monocytes on consecutive days. [12] These cells are known to produce cytokines, free radicals, metalloproteinases, nitric oxide and much more. Such substances directly harm the nerve tissue, induce apoptosis and damage the blood-brain barrier. The destruction to the BBB makes it permeable not only for leukocytes but also for water, which may cause the local edema. [13] Therefore, it has long been stressed that there is a connection between the severity of inflammation, the size of the area affected by ischemia and the degree of neurological deficit. [14] In this study, both the increased leukocyte count and the CRP on the first day of the disease were significantly associated with a more severe neurological status in the ultra-acute phase of the disease and with the functional status on the 30th day. Our results are consistent with previous reports and may be supportive of anti-inflammatory strategies in the treatment of stroke. [15] Studies on experimental stroke suggest that cytokines modulate the process of neuronal damage during ischemic stroke. Their effect on the evolution of infarct depends on their presence and the possibly on their concentration in the area of the penumbra. The interrelationship between ischemia and the activation of the inflammatory process is complex, and the division of acute stroke into stages of destruction and subsequent reconstruction is artificial, as these processes are most likely simultaneous. As cytokines take an active part in the evolution of the cerebral infarction zone, their measurement may serve as a marker of stroke severity in the acute phase of the disease and also as a marker of the outcome in later stages. [16,17] TNF-alpha, IL-1 beta, IL-6, and chemokines are the mediators of an early inflammatory response. [1]

Although TNF and interleukin 1, 2 and 6 are the most frequently studied cytokines in the CSF and blood of stroke patients, the conclusions are ambiguous and inconsistent with the results of experimental stroke studies. [2-4]

This study shows that a high TNF concentration on the first day of stroke correlates with a severe neurological status during that period, while a high IL-6 concentration is not only associated with the severity of neurological status during the early hours of stroke but also with a poor functional status on the 30th day of the disease. IL-6 expression was significantly higher in patients with symptomatic atherosclerosis in the coronary and/or carotid/cerebral arteries. There was no significant correlation between IL-2 and the clinimetric parameters during the first month of the disease. Experimental studies showed that TNF concentration is low after four hours of stroke, with a subsequent increase sustained for several days. [18] It is suggested that TNF has an unfavorable effect on the volume of stroke infarct, in contrast to the effect of IL-6, although there are also reports that contradict these relationships. Therefore, it is possible

that the effect depends on the concentration of these cytokines. [19-24] Regardless of individual reports on the neuroprotective potential of TNF and IL-6, just like reported by other investigators, we observed an adverse effect of high concentrations of these cytokines on the neurological status and the degree of post-stroke disability in the first month following onset. [25-28]

NSE and S100B are considered as biomarkers of glial cell damage or neuronal damage in patients with stroke or injury of the CNS. [29-37] Increased blood concentrations of NSE and/or S100B occur after hypoxia, hemorrhage, and injury to the CNS and always indicate that the BBB has been damaged. [38,39] We obtained a positive correlation between the presence of NSE and a worse neurological status on the first day, and between S100B and a poor status on the first and 30th day of stroke, which is consistent with the observations reported by other authors. [15, 40-42] We observed a positive correlation between the concentrations of NSE and S100B.

In recent years, researchers focused on progranulin (GRN), a glycoprotein growth factor with pleiotropic effects on the nervous system. Despite the results suggesting its protective role against ischemic brain injury, little is known about its importance for the prognosis. The results of experimental studies suggest that progranulin prevents BBB damage, causes neuroimmune suppression, is a regulator of vascular permeability, reduces the local brain edema and the size of infarct. In animal studies, it turned out to be a prognostic factor for motor functions. [43-44] In our study, we obtained a positive correlation between GRN concentration on the first day of stroke and the severity of the functional status on the 30th day of the disease. In studies conducted by Xie et al., GRN was a factor related to poor prognosis in post-stroke patients during a 6-month follow-up. [45] Although unfavorable early results were reported in clinical practice, there are experimental data related to the positive effect to reduce the risk of hemorrhagic transformation of an infarct lesion. [46]

The results of this study indicate that the VEGF and BDNF have an effect of summation. VEGF at low concentrations stimulates moderate angiogenesis and prevents delayed neuronal death while reducing the cytotoxic effect of glutamate, thereby increasing cell survival. [47-49] In addition, VEGF has a strong anti-inflammatory effect and promotes neuroplasticity, further enhancing the migration and proliferation of neuronal precursor cells. [50] However, a high concentration of VEGF promotes strong angiogenesis in the hypoxemic area, which can lead to the local edema and to a worsening of the prognosis. [51] Similarly, BDNF increases the

secretion of the anti-inflammatory cytokine, has a neuroprotective role in ischemic stroke, and stops inflammatory processes by modifying the MAPK pathway and Bcl-2 cascades. [52, 53]

The results of our study presented here show that the inflammation and damage to the nervous tissue and the BBB dominate in the acute period of cerebral ischemia, which is manifested by the expression of factors being active in the processes mentioned. Based on our observations, it seems that the above issues are important for the neurological status in the early hours of the disease and for the functional status one month after the onset. The expression of inflammatory agents is particularly exhibited in patients with symptomatic atherosclerosis. However, a novelty in our study is the demonstration of the primary role of the inflammatory state, whose parameters show a consistent effect on the degree of neurological deficit. We did not find a significant influence of the selected parameters of the angiogenesis or neurogenesis on the prognosis of patients' condition in the first month of the disease.

Regardless of the self-limiting nature of inflammation during acute cerebral ischemia, the use of modulators of this process may protect the nervous tissue from permanent damage and improve the prognosis for post-stroke disability.

Limitations:

The limitation of our study is the lack of analysis related to the relationship between the size of an ischemic lesion in head CT/MRI and the blood serum parameters selected for this project.

Conclusions

The concentration of IL-6 and S100B on the first day of stroke are significant for both the neurological status and the functional status in the acute period of the disease.

Increased CRP and leukocyte count are associated with a worse prognosis regarding the course of acute stroke.

The expression of pro-inflammatory agents and markers of blood-brain barrier damage in the acute phase of stroke is more prominent in patients with symptomatic atherosclerosis than in patients with no clinical features of atherosclerosis.

In the acute phase of stroke, there is a clear, positive correlation between factors of a similar profile of action.

The expression of inflammatory parameters indicates the importance of the inflammatory process, starting during the early days of ischemic stroke, for the post-stroke neurological deficit.

The Conflict of Interest Declaration.

The authors don't declare the conflict of interest.

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