Evaluation of novel factors influencing the outcome of ischaemic stroke

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PhD thesis

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ABBREVIATIONS

ACTH	adreno-corticotrop hormone
ADMA	asymmetric dimethylarginine
AIS	acute ischaemic stroke
BI	Barthel-index
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CD40L	CD40 ligand
CNS	central nervous system
DDAH	dimethylarginine dimethylamin-hydrolase
eNOS	endothelial nitric-oxid synthase
MCP-1	monocyte chemoattractant protein-1
hsCRP	high sensitivity C-reactive protein
ICAM-1	intracellular adhaesion molecule-1
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
mRS	modified Rankin scale
NIHSS	National Institute of Health Stroke Scale
NO	nitric-oxid
PCT	procalcitonin

PRMT	protein methyl-transferase
S100B	S-100-beta protein
SDMA	symmetric dimethyl-arginine
SIIDS	stroke-induced immundepression syndrome
TIA	transient ischaemic attack
TLR	Toll-like receptor
TNF-α	tumor necrosis faktor-α
TOAST	Trial of Org 10172 in Acute Stroke Treatment
tPA	tissue plasminogen activator
VCAM-1	vascular cell adhaesion molecule-1

I. INTRODUCTION

I/1. The epidemiology of ischaemic stroke

According to the World Health Organization (WHO) definition, stroke is a "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 hours or longer or leading to death, with no apparent cause other than of vascular origin". Stroke is the leading cause of minor and major long-term disability in adults and only 10% of the patients are able to live their previous way of life.

I/2. The prevalence of post-stroke infections

Not only the severity of the stroke itself, but the acute and chronic medical and neurological complications contribute to the mortality and poor functional outcome. Post-stroke infection is defined as an infection that occurs during the acute phase (0–7 days) of stroke. Post-stroke infections worsen the functional outcome and raise the mortality. The meta-analysis of 87 studies showed that infections affect approximately 30% of patients with acute stroke.

I/3. Preventive antibiotic treatment in acute stroke

Five randomised, placebo-controlled studies have examined the effect of preventive antibiotic treatment in stroke in recent years.

The Early Systemic Prophylaxis of Infection After Stroke (*ESPIAS*) study examined the effect of levofloxacin prophylaxis in patients with ischemic or hemorrhagic stroke. The study was stopped because levofloxacin did not decrease the rate infections.

Another antibiotic trial was the Preventive ANtibacterial THERapy in acute Ischemic Stroke (*PANTHERIS*). Patients were randomised to receive either intravenous moxifloxacin or placebo. Moxifloxacin decreased the rate of the post-stroke infections significantly but did not affect mortality and functional outcome.

In The Mannheim Infection in Stroke Study (MISS), patients received mezlocillin plus sulbactam. The combined antibiotic treatment decreased the rate of infection; and there was a trend of improved clinical outcome.

The results of the Preventive Antibiotics in Stroke Study (PASS) showed that although ceftriaxone therapy significantly prevented infections, it neither reduced mortality nor influenced the functional outcome.

The *STROKE-INF* study was published in 2015. Antibiotic prophylaxis did not decrease the rate of pneumonia and did not affect mortality and functional outcome. Thus, III.B. level evidences suggest that administration of preventive antibiotic treatment is not recommended in acute post-stroke period.

I/4. Central nervous system-injury induced immunodepression syndrome (CIDS)

After cerebral ischemia, the disruption of the blood-brain barrier allows leukocytes, including neutrophils, macrophages and lymphocytes to enter the damaged brain tissue and brain antigens to enter peripheral circulation. This process may expose brain epitopes which are normally "invisible" to the immune system and thus may promote priming and activation of lymphocytes reactive to central nervous system (CNS) antigens.

This crosstalk between the CNS and the immune system is partly mediated by the sympathetic nervous system, the hypothalamic-pituitary-adrenal axis (HPA) and the vagus nerve. The inflammatory cytokine production caused by brain tissue necrosis stimulates neurones in the paraventricular nuclei of the hypopthalamus (PVN) and leads corticotropin-releasing factor (CRF) production. Overactivation of the adrenergic system results in the release of catecholamines and corticosteroids. This model is supported by studies showing that increased serum cortisol and catecholamine levels in patients with acute stroke are independent predictors of mortality in stroke patients. The secretion of cortisol is regulated by ACTH, but in patients suffering from stroke, the level of cortisol is not related to the serum level of ACTH but rather to the IL-6. The increased amount of cortisol results in immunosuppression, which can increase the chance of infection. Due to the immunodepression and reduced pro-inflammatory immune responses, the susceptibility for infection is increased; indeed, post-stroke infection plays a major role in stroke-related mortality. On the other hand, CIDS may protect against damaging autoimmune responses elicited by exposed CNS antigens. This mechanism is called CNS-injury-induced immunodepression syndrome.

II. THE IMPACT OF POST-STROKE INFECTIONS ON THE OUTCOME OF ISCHEMIC STROKE

II/1. Aims of the study

First, we prospectively investigated the frequency of post-stroke infections in patients admitted with acute ischaemic stroke and their effect on functional outcomes and mortality. We also evaluated the etiology of ischemic stroke, the demographic data and co-morbidity of the patients, and their impact on post-stroke infections and outcomes.

II/2. Subjects and Methods

One hundred and six patients with acute ischemic stroke were enrolled within 6 hours after the onset of symptoms. Hemorrhagic stroke, infection and/or fever within <4 weeks, elevated white blood cell counts (WBC) and/or elevated PCT level on admission were exclusion criteria. Severity of stroke was measured by the National Institute of Health Stroke Scale (NIHSS). Medical history and demographic data were collected. Etiology of stroke was defined by TOAST criteria. Endpoints were appearance of post-stroke infection, mortality and functional outcome on the 28th day assessed by the modified Barthel index. Post-stroke infections were defined as infections that emerge within the 1st week after admission. Statistical analysis was performed by SPSS 16.0 software.

II/3. Results

II/3. a. Demographic data and concomittant diseases

The mean age of patients was 69.8 year, and women were 8 years older than men. Hypertension was the most frequent risk factor in both gender. The rate of smokers was five-times higher in men than women; on the other hand, the frequency of cardiac risk factors (atrial fibrillation and/or prostathic valve) were twice more common in women (**Table 1**).

Table 1. *Demographic data and concomittant diseases*

	Patients	**Women**	**Men**
N	108	57	51
Age (mean±SD)	69.79 (±12.01)	73.8 (±12.3)	65.77 (±10.26)
Hypertension (%)	71.6	68.2	76.5
Diabetes mellitus (%)	15.6	14	17.6
Smoking (%)	20.2	7	35.3
Previous stroke/TIA (%)	19.3	22.8	15.7
Cardiac risk factor (%)	37.6	49.1	25.5
Obesity (%)	12.8	14	11.8

II/3. b. The severity and outcome of acute ischemic stroke

The severity of stroke based on the NIHSS score at admission did not differ between men and women. The 7-day and emission NIHSS were significantly better in men than women. The 28-day functional outcome did not differ significantly between men and women (**Table 2)**

Table 2. *The severity and functional outcome of acute ischemic stroke*

	Mean (± SD)	**Women**	**Men**	***P-value*** **(women/men)**
NIHSS1	11.01 (±5.63)	11.9 (±5.52)	10 (±5.64)	0.887
NIHSS7	9.4 (±7.03)	10.8 (±8.04)	7.3 (±4.62)	***0.002***
NIHSS emission	10.39 (±13.96)	12.5 (±17.5)	7.32 (±12.5)	0.081
28-day BI	68.6 (±38.53)	64.7 (±34.9)	72.1 (±28.35)	0.225

Mann-Whitney U test; NIHSS1: National Institute of Health Scale score on day 1; NIHSS7: National Institute of Health Scale score on day 7; NIHSS emission: National Institute of Health Scale score on the day of emission;28-day BI:Barthel index on the 28th day after the onset of stroke

II/3. c. Classification of patients according to the TOAST criteria

Fifty-two percentage of patients suffered from cardioembolic stroke, 32% of patients had ischaemic stroke related to large-vessel atherosclerosis, and 16% of patients were admitted with lacunar stroke. Patients with cardio-embolic stroke were significantly older than patients in the other two groups (**Table 3**). The functional outcomes of stroke were significantly better in lacunar stroke compared with strokes from large-vessel atherosclerosis and cardio-embolic origin; there were no differences between the latter two groups (**Table 3**).

Table 3. *Age of patients and severity of stroke according to the TOAST criteria*

	Large-vessel athero-sclerosis	**Cardio-embolic**	**Lacunar stroke**	***P*-value large-vessel *vs.* cardio-embolic**	***P*-value large-vessel *vs.* lacunar**	***P*-value cardioemb. *vs.* lacunar**
age	68.7±10.03	77.3±9.48	66.4±10.25	***0.003***	0.814	***0.001***
NIHSS1	12.65±5.887	14.07±4.89	6.89±3.71	0.506	***0.003***	***0.000***
BI 28 day	60±30.1	61.3±36.68	85.3±19.2	0.694	***0.024***	***0.020***

Mann-Whitney U test; NIHSS1: National Institute of Health Scale Score on day 1; BI 28 day: Barthel index on the 28th day after stroke onset

II/3. d. Post-stroke infections and mortality

Twenty-one percentages of our patients suffered from post-stroke infection affecting 8% of men and 34% of women. Cardioembolic stroke and obesity showed the strongest correlation with the appearance of post-stroke infections. Twenty-one patients (4 men and 17 women) died: mortality significantly correlated with the severity of stroke. Occurence of infections significantly correlated with the age of patients, the NIHSS score on admission, the 7th day NIHSS score and NIHSS score at discharge, but did not correlate with functional outcome on the 28th day. Mortality showed correlation with the appearance of post-stroke infection.

II/4. Discussion

The mean age of the 106 patients admitted with acute ischaemic stroke weas 69.7 year, and women were 8 years older than men. Hypertension was the most frequent risk factor in both gender groups. The rate of smokers was 5-times higher in men than women, but cardiac risk factors appeared twice in women (25% vs 50%). Strokes of large-vessel disease and cardioembolic origin were significantly more severe than lacunar stroke. The severity of stroke had a crucial part in mortality. Post-stroke infections - affecting 20% of patients - significantly correlated with age, obesity, severity of stroke and cardio-embolic origin. In case of infections, the mortality was significantly higher.

Hospital-based data in almost 20000 stroke patients obtained from the Swedish Stroke Registry showed that the incidence of atrial fibrillation increases with age, and women were 4 years older than men. Strikingly, our male patients were 10 years younger and female patients 5 years younger than Swedish patients with stroke. Data from the Swedish registry showed that the frequency of cardio-embolic stroke did not differ according to gender. This is substantively different data what we observed: the rate of cardioembolic stroke was twice more frequent among women than men in our cohort. In the Framingham Heart study, age was one of the strongest risk factor for having atrial fibrillation. Since our women patients were 8 years older than men, it can be an explanation, why the rate of cardioembolic stroke was much higher among women. Twenty percents of our patients had post-stroke infection similar to published data. Infections affected women four-times more than men, and appearance of infection significantly correlated with mortality. Since stroke with cardioembolic origin was twice more frequent among women, and it was the most severe, it may explain, why the mortality of women

was four-times higher. Among patients, who survived the acute post-stroke period, post-stroke infections did not influence the 28th day functional outcomes.

III. BIOMARKER MEASUREMENTS AND THE FUNCTIONAL OUTCOME OF ISCHAEMIC STROKE

III/1. Aims of the study

Here, the temporal profile of 8 biomarkers, which contribute to thrombo-inflammatory process, endothelial dysfunction and systemic immune responses, or correlate with infarct size were prospectively investigated in 77 patients with acute ischaemic stroke. We examined their baseline levels, their concentration 72 hours later, the temporal change of the biomarker concentration, and the predictive potential of all these factors for stroke outcomes. The primary endpoints were mortality, occurance of post-stroke infection and functional outcome on the 28th day. We examined the simultaneous relation of the measured biomarkers to the outcomes (death, infection, function) using multiple regression, and examined the regression models' ability to predict the different outcomes.

III/2. Subjects and Methods

Seventy-six patients with acute ischemic stroke were enrolled within 6 hours after onset of symptoms: medical history was collected; standard laboratory tests, 12-lead ECG, and cerebral CT were performed. Infection and/or fever within <4 weeks and elevated white blood cell counts on admission were exclusion criteria. Severity of stroke was measured by the National Institute of Health Stroke Scale (NIHSS). NIHSS was measured daily till day 7. The etiology of stroke was defined by the TOAST criteria. The outcome was assessed by the modified Barthel index (BI) on the 28th day. Thirteen patients received intravenous thrombolysis. The size of infarct was assessed by the concentration of S100-beta protein in the serum (S100B). Levels of 7 additional biomarkers were measured that may all contribute to thrombo-inflammation, endothelial dysfunction and systemic/local inflammation: hypersensitive C-reactive protein (hsCRP), CD40-ligand (CD40L), tissue plasminogen activator (tPA), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and P-selectin. Baseline and 72-hour levels were measured and the change of concentrations was calculated.

Two control populations were used: 44 patients with significant carotid stenosis (70-100%) determined by Duplex scan sonography but without acut vascular events, to determine if concentrations are related to the acute ischemic event itself or are inherent to the pre-existing atherosclerosis; and 66 patients with Parkinson disease as non-vascular controls.

Next, we prospectively analysed the rate of infectious complications during hospitalization and its relationship with the concomittant diseases and demographic factors. We analyzed, whether the severity of stroke measured by NIHSS score and serum S100 beta titer influence the prevalence of post-stroke infections. Finally, we simultaneously considered the 8 biomarkers (hsCRP, S100B, CD40L, tPA, MCP-1, IL-8, IL-6, P-selectin), using their absolute concentrations at different time points (values at baseline and at 72 hours) and the change from

baseline to 72 hours as covariates. Their association with post-stroke infections, death and functional outcome were investigated. We applied multiple regression methods, relating biological biomarkers combined with demographic data and co-morbidities to the considered outcomes. For the analysis of NIHSS at day 7, we also added the value of NIHSS at day 1 as covariate, and assessed the ability of the models to predict each outcome.

III.3. Biomarkers

III/3 a. S100beta protein

S100B protein is a member of a group of small Ca^{2+}-binding modulators. Members of this protein family have been implicated in the Ca^{2+}-dependent regulation of a variety of intracellular activities, such as protein phosphorylation, enzyme activities, cell proliferation and differentiation, intracellular Ca^{2+} homeostasis, inflammation, and protection from oxidative cell damage. Several studies have shown that serum S100B levels measured in serum samples taken more than 24 h after stroke onset demonstrate a strong correlation with the degree of neurological deficit and the final infarct volume. One metaanalysis published in 2012 showed a strong correlation between the brain tissue damage certified by radiological investigations and the value of S100B 48-72 hours after brain tissue damage. Therefore, we used this marker to measure the infarct volume indirectly.

III/3. b. hsCRP

High sensitivity C-reactive protein is a member of acute phase proteins. Recently, it was shown that elevated hsCRP levels independently predicted future stroke and transient ischemic attack in elderly. More than 20 prospective studies of distinct cohorts have demonstrated that increased hsCRP concentrations are associated with an increased risk of cardiovascular events after multivariate adjustment for traditional risk factors.

III.3. c. Interleukin 6 (IL-6)

IL-6 promotes the expression of adhesion molecules, activation of leukocytes, and results in a prothrombotic state. The increased concentration of IL-6 in the serum is a characteristics of acute ischaemic stroke, and the level of IL-6 correlates with the infarct volume and the outcome of stroke.

III./3. d. Monocyte-chemoattractant protein-1 (MCP-1)

MCP-1 is a member of chemokines that control the migration of neutrophils, lymphocytes, antigen-presenting cells including dendritic cells, and cells of the monocyte/macrophage lineage. There is strong evidence that MCP-1 is involved in the recruitment of both monocytes, macrophages and activated lymphocytes into the CNS.

III.3. e. Soluble CD40 ligand (sCD40L)

Soluble CD40 ligand is a transmembrane protein with pleiotrop effects expressed by antigen-presenting cells. The engagement of the CD40 and CD40 ligands regulates a wide spectrum of molecular and cellular processes, including the initiation and progression of cellular and humoral adaptive immunity.

III/3. f. P-selectin

P-selectin is produced by both platelets and endothelial cells, and mediates early inflammatory cell adhesion. The ligand of P-selectin is expressed mainly by polymorphonuclear cells and monocytes. Such intercellular communication results in responses by all these cell populations: leukocyte invasion, enhanced platelet aggregation and thromboxane release. Experimental data showed that anti-P-selectin monoclonal antibody therapy significantly decreases the leukocyte infiltration to brain tissue and the volume of the infarction in case of cerebral media artery occlusion.

III/3. g. Tissue plasminogen activator (tPA)

Tissue plasminogen activator is a protein involved in the breakdown of blood clots. It is a serine protease found on endothelial cells. As an enzyme, it catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for clot breakdown. Therapy with recombinant tPA in selected patients with acute ischaemic stroke is I.A level of evidence.

III.3. h. Interleukin-8 (IL-8)

Interleukin-8 induces the migration and phagocytosis of leukocytes. Experimental data showed that IL-8 receptor blocker reparixin decreased the brain infarct volume, and ameliorated the neurological outcome in case of middle cerebral artery occlusion.

III/4. Results

III/4. a. Biomarker concentrations in the hyperacute phase of ischemic stroke (within 6 hours)

Biomarkers in the sera were examined within 6 hours after the onset of acute ischemic stroke: systemic level of all biomarkers but IL-8 were higher compared to both control groups, PD and significant carotid stenosis. Concentrations were also higher in patients with carotid stenosis compared to PD, except P-selectin.

III/4. b. Temporal profile of biomarkers in the acute phase of ischemic stroke

Changes in biomarker levels during the acute phase of ischemic stroke were also examined (within 6, 24 and 72 hours). Three temporal profiles were observed:

(i): elevation at 6 hours with additional significant increase by 72 hours (IL-6, P-selectin)

(ii) elevation at 6 hours and significant decline thereafter (IL-8)

(iii) decreasing value by 24 hours and constant elevation thereafter by 72 hours (tPA, CD40L and MCP-1), but this elevation was not significant.

III/4. c. Correlation with the size of infarct

To evaluate the infarct size, we measured the concentration and temporal change of S100B int he serum, and correlated its concentration with levels of biomarkers.

(i) Levels of hsCRP positively correlated with S100B level at any timepoint and the changes of its value.

(ii) Two other biomarkers showed correlation with S100B concentrations: both baseline and 72-hour IL-6 level correlated with S100B levels at 72 hours. The 72-hour IL-6 values correlated with the change of S100B.

(iii) Concentration of IL-8 at 72 hours positively correlated with change of S100B titers.

(iv) Baseline P-selectin concentration correlated with the change of S100B titers (**Table 4**).

Table 4*. Correlation of S100Bconcentration reflecting the size of infarct with concentration of biomarkers*

Biomarkers		S100B		
		6 h	**72 h**	**Δ**
		***P*-value**	***P*-value**	***P*-value**
hsCRP	6 hour	0.06	0.06	0.40
	72 hour	***≤0.001***	***≤0.001***	***0.003***
	Δ	***≤0.001***	***0.005***	0.11
tPA	6 hour	0.22	0.06	0.93
	72 hour	0.36	0.23	0.93
	Δ	0.88	0.80	0.25
P-selectin	6 hour	0.15	0.50	***0.04***
	72 hour	0.84	0.25	0.19
	Δ	0.34	0.91	0.15
MCP-1	6 hour	0.51	0.17	0.95
	72 hour	0.31	0.66	0.45
	Δ	0.10	0.10	0.43
IL-8	6 hour	0.86	0.10	0.48
	72 hour	0.08	0.36	***0.02***
	Δ	0.21	0.16	0.68
IL-6	6 hour	0.09	***0.003***	0.19
	72 hour	0.42	***0.02***	***0.04***
	Δ	0.80	0.35	0.16
CD40L	6 hour	0.65	0.55	0.30
	72 hour	0.65	0.32	0.07
	Δ	0.05	0.70	0.19

Significance levels determined by Spearman's non-parametric correlations are shown. Δ: change of concentration between post-stroke 6 and 72 hours

III/4. d. Biomarker levels according to TOAST criteria

It was ascertained that:

(i) None of the biomarker level differed significantly between the patients with large-vessel stroke and cardioembolic strokes at any time-points.

(ii) Baseline IL-6 level and 72-hour hsCRP and S100B values significantly differed between lacunar stroke and both large-vessel and cardioembolic stroke patients. Similarly, the 72-hour P-selectine level in large-vessel and cardioembolic group was signifiantly higher than the values measured in lacunar stroke cases.

III/4. e. Correlation with post-stroke infection and death

Next, we examined, which biomarker level or its change were associated with post-stroke infection and death.

Biomarker levels correlated with *mortality*:

, (i) baseline and 72-hour hsCRP value and its change

(ii) the change in the level of P-selectine and CD40L between 6 and 72 hours

(iii) baseline levels of IL-6 and IL-8

(iv) baseline and 72-hour levels of S100B

Biomarker levels correlated with *post-stroke infections*:

(i) levels of hsCRP measured at 72 hours and its changes between the two time-points

(ii) changes of P-selectine levels

(iii) baseline and 72-hour levels of IL-6 and S100B and their changes

III/4.f. Regression analysis

For the three outcomes (death, post-stroke infections and 28^{th}-day functional outcome), we established a regression model to relate each outcome to the measured biomarkers, and in order to control for possible confounders. We simultaneously considered the 8 biomarkers using their values at baseline, at 72 hours and the change from baseline to 72 hours as covariates. In addition, demographic/comorbidity variables were also included: age, sex, smoking, obesity (BMI), and diabetes. For the analysis of NIHSS at day 7, we also added the value of NIHSS at day 1 as covariate.

Outcome 1: death:

(i) each additional year of age increased the odds by 31%;

(ii) each unit of hsCRP at baseline increased the odds by 7%;

(iii) each 100 units of IL-6 at baseline increased the odds by 9‰

Outcome 2: post-stroke infections:

(i) female sex increased the odds on average by a factor of 15.3;

(ii) elevation in P-selectin concentration between 6 and 72 hours by a factor of 22.7;

(iii) each additional 100 units of IL-6 at 72 hours by 4‰.

Outcome 3: NIHSS score by day 7 was increased:

(i) with 0.9 points on average by each NIHSS point measured on day 2;

(ii) with 2.5 points, when baseline concentration of IL-6 in the peripheral blood was measurable;

(iii) with 2.1 points by obesity;

(iv) with 0.2 points by each year of age;

(v) with 1.6 points by male sex.

III/5. Discussion

Here, we prospectively investigated 8 biomarkers in acute ischemic stroke, which have the potential to contribute to thrombo-inflammation in the very early phase, and thus influence the outcome.

Compared to patients with PD, systemic concentration of tPA, P-selectin, MCP-1, IL-8, IL-6 and CD40L were elevated both within 6 hours after stroke and 72 hours later. Therefore, we compared data to patients with severe asymptomatic carotid stenosis, and we also examined the temporal profiles.of the biomarkers. Concentration of all biomarkers but IL-8 was higher within 6 hours compared to patients with carotid stenosis, suggesting that increased levels were not related to the atherosclerosis per se. However, concentration of tPA, MCP-1, IL-8, IL-6 and CD40L were also higher in patients with carotid stenosis compared to PD, indicating that severe atherosclerosis was associated with increased systemic concentrations, and stroke resulted in an additional acute increase. We used multiple regression to examine, which combination of factors could best predict post-stroke infection, death and NIHSS score by day 7; as covariates, we considered biological biomarkers and their changes, demographic data and co-morbidities. Particularly, change of P-selectin from 6 to 72 hours by one unit increased the incidence of post-stroke infections with an OR of 22.7; each 100 units of IL-6 at baseline increased the odds

of death by 9‰ and IL-6 level at 72 hours increased the odds of post-stroke infections by 4‰; each unit of baseline hsCRP elevated the odds of death by 7%.

Interestingly, P-selectin, IL-6, and hsCRP contributing to outcomes in our models, all showed similar temporal profiles: an additional elevation by 72 hours besides an increased concentration on admission (within 6 hours) compared to both patients with carotid stenosis and PD.

P-selectin is produced by both platelets and endothelial cells, and mediates early inflammatory cell adhesion. Thus the early elevation of P-selectin may be associated with endothelial dysfunction and platelet activation, whereas the later increase may reflect leukocyte invasion peaking around 72 hours. Experimental data showed that anti-P-selectin monoclonal antibody therapy at permanent middle cerebral artery occlusion decreased the invasion of leukocytes to the damaged brain tissue, and decreased the volume of the infarction. However, since change in P-selectin levels correlated with post-stroke infections in our study, it is also possible that late elevation indicated leukocyte activation related to an early, subclinical phase of infection.

The role of IL-6 in stroke is somewhat controversial. Upregulation of IL-6 on neurons, glial cells and vascular endothelium is a consistent finding in animal models of cerebral ischemia. Although IL-6 is also elevated in the CSF of patients with stroke, its cellular source is unclear: it can reflect systemic release and passive passage due to the blood-brain barrier disruption, or release from dying neurons and production by microglia. IL-6 on admission correlated with concentration of S100B at 72 hours in our cohort, indicating that early systemic production of IL-6 correlates with the infarct volume on biomarker levels, similar to neuroimaging studies. Higher level of IL-6 after stroke also correlated with early neurological deficit, body temperature and long-term poor outcome in previous studies. It is likely that alterations in systemic and intrathecal levels of IL-6 may reflect different cellular sources elicited by different interactions/pathways, and eventually contribute to the differential effects. Early systemic elevation may thus be related to thrombotic events and platelet-endothel-monocyte interactions, while late elevation can indicate leukocyte activation. Leukocyte infiltration and local activation within the ischemic tissue may be deleterious, but proper systemic activation of leukocytes is required to protect against infections in the post-stroke period.

Similarly, different causes may contribute to elevation of hsCRP at 6 and 72 hours, and late increase in concentration may be related to a subclinical infection. This may also explain, why an increase of hsCRP by 72 hours correlates with post-stroke infections. Correlation between S100B and hsCRP levels may also point to the fact that patients with more extensive infarcts are more susceptible to post-stroke infections due to more profound immune dysfunction.

Several available data show that the presence of post-stroke infections increases the mortality of stroke significantly. Therefore it seems logical to use antibiotic prophylaxis in the acute phase of ischaemic stroke to prevent infections.

However, preventive antibiotic trials were controversal. But the STROKE-INF study published in 2015 provided II.B. evidence that preventive antibiotic treatment in acute stroke is inappropiate. Most probably, not only the pathogenic microorganism itself but other

predisposing factors may have a potential role in the development of post-stroke infection. I the recent years, a lot of attention have been focused on the role of the innate and humoral immune responses, and the CNS injury induced immuno-depression syndrome concept has been accepted.

At present, immunomodulatory therapy is in the centre of interest in acute stroke research (**Table 5.)**

Table. 5. *Studies of immunmodulatory therapies in acute stroke*

Drug	Mechanism	Stroke onset	Duration of treatment	Phase	Clinical, radiological outcome	Summary
Enlimomab	anti-ICAM-1 monoclonal At	0-6 h	5 days	III n: 625	SAE, mRS score mortality, infarct volume	Effective in experimental stroke, clinical trial terminated
rh IL-1a	IL-1 receptor antagonist	1-4 months	30 day	II n=34	Safe, well tolerated, decreased the mRS score	Safe, effective
Minocyclin	early modulation of inflammatory signals	6-24 h	5 days	Open-label n=152	Safe, well tolerated, decreased the mRS score	Effective in experimental stroke, clinical trial terminated
Fingolimod	sequesters lymphocytes in lymph nodes, preventing an autoimmune reaction.	0-4.5 h, combined with alteplase	3 days	Open-label n=47	Safe, well tolerated, decreased the mRS score and the volume of infarct	Decreased the reperfusional injury, improved functional outcome
Natalizumab	£4-integrin blocker	0-9 h	single dose	II n=200	NA	ongoing

ICAM-1: intracellular cell adhaesion molecule; rh-IL-1a: recombinant human IL-1 antagonist; SAE: severe adverse event; mRS: modified Rankin scale

In our cohort, the change in the concentration of P-selectin was predictive for forthcoming post-stroke infection. We contemplate that it may be useful to early identify patients with the highest risk for CIDS and infection. These patients may then benefit from novel immunomodulatory therapies being currently in the focus of stroke research.

IV. THE L-ARGININE PATHWAY IN ACUTE ISCHAEMIC STROKE AND SEVERE CAROTID STENOSIS

IV/1. The role of L-arginine metabolites

Asymmetric-dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are protein breakdown products of L-arginine. ADMA directly inhibits endothelial nitric oxide synthase (eNOS), thus reduces NO production, whereas SDMA competes with the NO precursor arginine for uptake into endothelial cells. Numerous data support the beneficial effect of eNOS and vascular NO in the early stages of cerebral ischemia playing a prominent role in maintaining cerebral blood flow and preventing neuronal injury. Indeed, eNOS knockout mice show decreased blood flow in the ischemic border zone and develop larger cerebral infarctions. Although prognostic value of serum concentrations of methylated arginine derivatives has not been fully elucidated in stroke, an increase in both ADMA and SDMA plasma levels within the first 72 hours after the onset of ischemic stroke may predict poor outcome.

IV/2. Aims of the study

The aims of this study were the following:

(i) investigation the L-arginine pathway in patients with asymptomatic significant carotid stenosis and acute ischemic stroke compared with age-matched healthy controls;

(ii) longitudinal change of the L-arginine metabolites during the acute phase of acute ischemic stroke;

(iii) correlation of the L-arginine pathway with biomarkers reflecting infarct size and infection;

(iv) Predictive value of the L-arginine metabolites on death and post-stroke infections.

IV/3. Patients and Methods

A total of 55 patients suffering from acute ischemic stroke, and 44 patients with asymptomatic significant carotid stenosis were prospectively selected for this study. Forty-five age-matched healthy blood donors served as normal controls. Medical history concerning previous coronary artery disease, chronic obstructive pulmonary disease, diabetes, hypertension, current cigarette smoking, serum creatinine levels, and body mass index was obtained from each patient. Definitive stroke was defined according to international guidelines. Venous blood samples from patients with stroke were taken serially for measurement of ADMA, SDMA, L-arginine, hsCRP, and S100 B within 6 hours after the onset of first symptoms, and at 24 and 72 hours.

IV/4. Results

IV/4. a. Baseline levels of metabolites within 6 hours after stroke

Concentration of all 3 metabolites (L-arginine, ADMA, and SDMA) within 6 hours after the onset of ischemic stroke was significantly higher in the sera compared with healthy controls and patients with significant carotid stenosis. SDMA was also lower in the sera of patients with carotid stenosis and healthy contols.

IV/4. b. Temporal profile of L-arginine metabolites in acute ischemic stroke

We investigated changes in the serum concentrations of L-arginine, ADMA, and SDMA within 6 hours after onset of AIS as well as at 24 and 72 poststroke hours. We observed a slight increase in the concentration of L-arginine at 24 hours in contrast to a slight decrease in the levels of ADMA; the concentration of SDMA increased continuously. The ratio of L-arginine/ADMA was significantly increased at 24 hours and decreased thereafter, whereas we observed a decrease in L-arginine/SDMA ratio (**Figure 1)**.

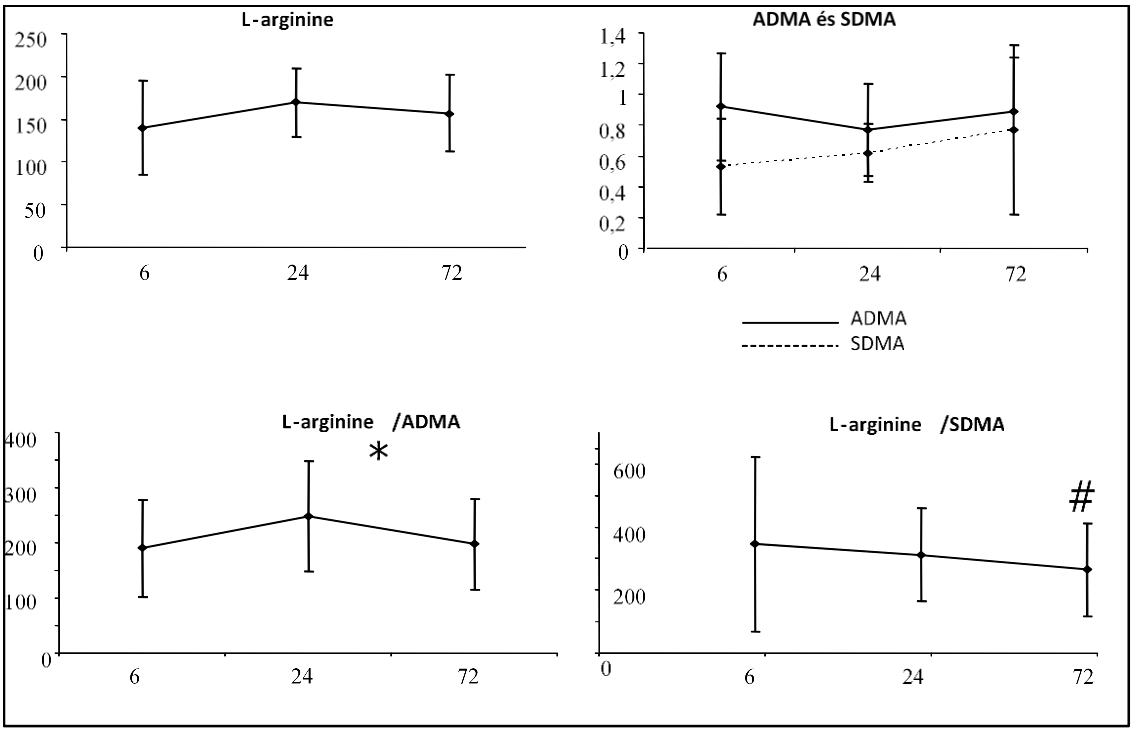

Figure 1. *Temporal profile of L-arginine and methylarginine derivatives in acute ischemic stroke. Temporal profile of L-arginine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and L-arginine/ADMA and L-arginine/SDMA ratios respectively measured at 6, 24, and 72 poststroke hours. Data are presented as mean ± standard deviation. *indicates significantly higher L-arginine/ADMA ratio at 24 hours compared with 6 hours. #indicates significantly lower L-arginine/SDMA ratio at 72 hours compared with 6 hours.*

IV/4. c. Correlation of L-arginine metabolites with S100B protein

Next, we examined if concentration of metabolites of the L-arginine pathway correlates with S100B reflecting infarct size. Concentration of S100B positively correlated with the level of SDMA at 72 post-stroke hours. Beside the absolute concentrations, we also investigated whether the change of concentration of S100 B within 72 hours showed any correlation with changes in the concentration of the 3 metabolites. Although absolute concentrations of ADMA did not correlate with the corresponding S100B concentrations, changes in concentration of ADMA by 72 hours did show a positive correlation with changes in the concentration of S100B.

I/4. d. Correlation of L-arginine metabolites with C-reactive protein

We also examined correlation of the L-arginine pathway with concentration of hsCRP both in hyperacute and subacute phase of AIS, as well as in patients with carotid stenosis. Concentration of hsCRP within 6 hours after the onset of stroke negatively correlated with L-arginine levels. Concentration of hsCRP at 72 post-stroke hours positively correlated with the level of SDMA.

IV/4. e. Prediction of death and post-stroke infection

None of the metabolites of the L-arginine pathway were predictors of death. Multiple logistic regression including all variables indicated that changing levels of SDMA and hsCRP were predictive for post-stroke infections.

V. Discussion

We observed that concentrations of all 3 metabolites (L-arginine, ADMA, and SDMA) were elevated in the very acute phase of ischemic stroke. An increased basal L-arginine level in patients with sichemic stroke might be an adaptive mechanism. L-arginine is the precursor molecule for NO, and NO produced by eNOS plays a crucial role in the regulation of vascular tone resulting in vasodilation. The extracellular arginine availability may have a beneficial influence on cerebral perfusion by increasing NO. It was published in 2013 that increased L-arginine/ADMA ratio in patients with subarachnoidal hemorrhage correlated with improved functional outcome and decreased mortality. Authors suggested that increased blood supply of the brain can explain their observations. On the other hand, increased levels of ADMA did not correlated with vasospasm detected by transcranial Doppler sonography. They interpreted that ADMA causes unfavoruable conditions in the microcirculation. We observed transient increasing ratio of L-arginine/ADMA, and this transient elevation of L-arginine may be part of compensatory mechanisms, which result in vasodilatation in the damaged brain tissue. Experimental data suggested that parenteral L-arginine supplementation increased the eNOS-dependent cerebral blood perfusion. In our cohort, we found that neither the absolute levels of ADMA nor the change in ADMA levels were predictive for stroke outcome and the presence of post-stroke infections.

Less information is available about the role of SDMA in cardiovascular diseases. In our study, the changing concentration of SDMA was predictive for post-stroke infections. It was published in 2012 that concentration of SDMA correlated with adverse clinical outcome during the first 30 days after ischemic stroke. In a large multiethnic population-based cohort, SDMA but not ADMA was an independent predictor of cardiovascular mortality.

Next, we examined the relationship between the L-arginine pathway and 2 biomarkers: (i) S100 B, which correlates with the size of infarct, and (ii) hsCRP, which is regarded as both an atherosclerosis and an infection marker.

We show that the concentration of L-arginine inversely correlates with the early elevation of hsCRP. This may suggest that L-arginine indeed may play a protective role, and low L-arginine levels are associated with a higher concentration of hsCRP, a risk factor for stroke. We also observed that concentration of S100B reflecting the infarct size correlates positively with SDMA at 72 poststroke hours, and change of S100B also positively correlates with concentration of ADMA. These data may suggest a relationship between the infarct size and L-arginine pathway. We also examined the effect of L-arginine pathway on post-stroke infections. Change in SDMA concentration by 72 hours was an independent predictor of post-stroke infections.

In short, we can conclude that elevated levels of L-arginine metabolites in the acute phase of ischemic stroke indicate enhanced endothelial dysfunction. The early increase in the level of L-arginine may be an adaptive reaction for this vascular dysfunction. Increasing level of SDMA can be also a predictor of forthcoming post-stroke infections.

It is debatable, whether therapeutic interventions influencing the levels of L-arginine metabolites can improve endothelial dysfunction and clinical outcome. Modulators of the L-arginine pathway are the statins, angiotensin-converting enzyme inhibitors, and isoform-specific NOS inhibitors. On the other hand, the elevated levels of L-arginine metabolites can be also consequences of an irreversible tissue damage. Considering these, further studies are required.

VI. SUMMARY OF THE THESIS

1. Elevated hsCRP levels in the hyperacute phase of ischaemic stroke correlated with the severity of stroke.

2. Increased concentration of hsCRP 72 hour after stroke correlated with forthcoming post-stroke infections.

3. S100B level at 6 and 72 hours reflecting the volume of brain infarct correlated with the occurrence of post-stroke infections and mortality.

4. IL-6 level both at 6 and 72 hours was predictive for unfavourable outcome and post-stroke infections.

5. Changing levels of P-selectin between 6-72 hours was highly predictive for post-stroke infection.

6. The L-arginine, ADMA, SDMA concentrations are elevated in hyperacute phase of stroke indicating severe endothelial dysfunction.

7. L-arginine level (already decreasing) measured at 72- hour and simultaneusly increasing SDMA concentrations are early predictors of post-stroke infections.

VII. PUBLICATIONS

Publications related to the Thesis

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