Identification of novel predictive biomarkers in patients with aneurysmal subarachnoid haemorrhage

DOCTORAL THESIS

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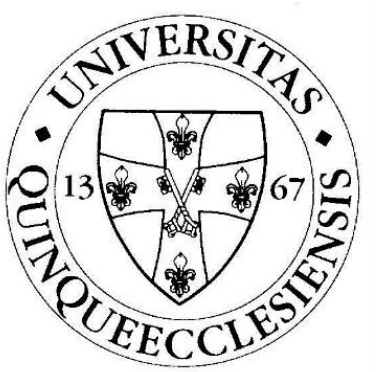

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1. Introduction

Acute stroke is a leading cause of death and one of the most common causes of permanent disability worldwide. In our country around 26,000 stroke cases occur each year, placing a significant burden on both healthcare and society, the two major categories of stroke (hemorrhage and ischemia) are the opposite conditions. Ischemic stroke accounts for approximately 80-85%of all stroke cases, while hemorrhage is responsible for the remaining 15-20%. Each of these categories can be divided into subtypes that have somewhat different causes, clinical pictures, clinical courses, outcomes, and treatment strategies. Intracranial hemorrhage can be caused by intracerebral hemorrhage (ICH, also called parenchymal hemorrhage), which involves bleeding into brain tissue, and subarachnoid hemorrhage (SAH), which involves bleeding into the cerebrospinal fluid that surrounds the brain and spinal cord and subdural, epidural hemorrhage.

The incidence of aneurysmal SAH (aSAH) varies widely by geographic region. The overall incidence of aSAH is approximately 10/100 000 person /year (about 1000 case in a year) in Hungary belonging to the average region. The mean age of aneurysmal rupture is in the range of 50 to 55 years, mainly in the fifth and sixth decade. The preponderance of women starts only in the sixth decade. The decline in incidence of SAH over the past 45 years is relatively moderate compared with that for ischemic stroke in general.

SAH can be divided into traumatic and spontaneous subgroups. Traumatic contusion is the most common subtype, but the majority of spontaneous SAHs are caused by ruptured saccular aneurysms (75-80%). Majority of intracranial aneurysms (approximately 85 percent) are located in the anterior circulation, predominantly on the circle of Willis. Common sites include the junction of the anterior communicating artery with the anterior cerebral artery, the junction of the posterior communicating artery with the internal carotid artery, and the bifurcation of the middle cerebral artery.

Hypertension, cigarette smoking, and connective tissue diseases such as Ehlers-Danlos syndrome, autosomal dominant polycystic kidney disease probably play a substantial role in the process of aneurysm formation, interestingly there is some evidence that inflammation plays a pivotal role in the pathogenesis and growth of intracranial aneurysms.

Most intracranial aneurysms are asymptomatic unless they rupture, and so they are usually found either incidentally or when a patient presents with SAH.

The classic presentation of patients with aSAH is a sudden-onset, severe headache typically described as the "worst headache of my life". In addition to headache, common

associated symptoms include a brief loss of consciousness, hypnoid alteriation of consciousness, nausea, vomiting, neck pain or stiffness, photophobia, ocular bleeding and diverse focal neurological signs, including third and sixth nerve palsy, ophthalmoplegia, hemiparesis, aphasia, neglect.

The cornerstone of SAH diagnosis is the noncontrast head computer tomography (CT) scan. If there is a strong suspicion of SAH despite a normal head CT lumbar puncture is mandatory. CT angiography is a cost effective and sensitive option in the initial identification of bleeding source. CTA has a reported a sensitivity of 97%–100% for the detection of intracranial aneurysms. Digital Subtraction Angiography (DSA) is the preferred next step in the management of patients with aSAH, since vascular malformations can be analyzed under higher resolution and at the same time endovascular treatment can be performed.

After aneurysmal SAH, there is high risk of rebleeding. Aneurysm repair with surgical clipping or endovascular coiling is the only effective treatment to prevent this occurrence and should be performed as early as feasible, preferably within 24 hours. Anatomic considerations, such as size, location, along with other morphological features determine which treatment is most appropriate for the patient. According to the recently published results endovascular coiling is preferred over clipping, considering higher rate of good functional outcome even after 7 years follow-up in the endovascular group.

Complications are common after SAH and contribute substantially to the overall morbidity and mortality. First off, all, rebleeding is associated with high mortality. Aneurysm treatment via endovascular coiling or neurosurgical clipping is the only effective treatment.

Delayed cerebral ischemia (DCI) occurs in about 30 percent of patients with aSAH, typically between 4 and 14 days after symptom onset. The definition of DCI requires the occurrence of focal neurologic impairment (such as hemiparesis, aphasia, apraxia, hemianopia, or neglect) or a decrease of at least two points on the Glasgow Coma Scale that lasts for at least one hour, was not apparent immediately after aneurysm occlusion, and cannot be attributed to other causes after appropriate clinical assessment, brain imaging, and laboratory studies.

In the last decade cerebral vasospasm (CV) was assumed to be the only trigger of DCI. Recently some other mechanisms have been revealed other than CV, such as vascular dysfunction, neuroinflammation, and spreading depolarization, which may contribute to DCI.

Clinical trials clearly demonstrated that targeting vascular dysfunction through vasodilation alone is not sufficient to reduce DCI.

Hydrocephalus and epileptic seizures belong to the severe complications after SAH as well.

Several scales have been established in the context of aSAH to estimate severity and prognosis. World Federation of Neurosurgeons scale (WFNS) is based on the assessment of the Glasgow Coma Scale and the presence of severe focal neurologic deficits. The mFisher scale classify the severity based on native CT scan, while localization, ventricular involvement, bleeding expansion is assessed.

Until these days no reliable prehospital prognostic factor has been discovered. One potentially encouraging method is the measurement of specific serum biomarkers. However, there is a use of limitation of recently explored blood biomarkers in the care of patients with aSAH.

Neuron and astrocyte specific markers, including S100β, neuron-specific enolase (NSE), glial fibrillary acid protein (GFAP), apolipoprotein E (apoE) have been studied as a prognostic adjunct tool, monitoring outcome following aSAH. Furthermore, biomarkers of the clotting cascade (Vascular endothelial growth factor (VEGF), Endothelin-1, von Willebrand factor (vWf)) and inflammatory markers [C-reactive protein (CRP), TNF-α, IL-1] have been investigated. However, no biomarker has been found yet, that would be able to predict the outcome in aSAH patients with high specificity and sensitivity.

The onset of aSAH elicits the activation of the thrombo-inflammatory cascade, causing ongoing neuroinflammation that is suspected to contribute to complications, such as CV and DCI. A number of thrombo-inflammatory markers have been investigated in the context of cerebrovascular pathology

Tumor necrosis factor superfamily-14 (TNFSF14) or LIGHT (homologous to lymphotoxin, exhibits inducible expression and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator [HVEM], a receptor expressed on T cells), a new member of the TNF superfamily, is a 29 kDa type II transmembrane protein produced by activated T cells. LIGHT/TNFSF14 has been linked to a number of diseases such as multiple sclerosis, inflammatory bowel diseases, graft vs. host disease and atherosclerosis. It can either costimulate or restrict the immune response binding to a subset of TNF superfamily receptors through diverse mechanisms. Members of TNF superfamily play an important role in immunity and activation, proliferation, differentiation, or even migration of immune cells into the central nervous system, serving as possible target in CNS autoimmune disorders like multiple sclerosis. Several members of the TNF superfamily have been recently emerged in animal models of SAH. In addition, TNFSF12, also known as TWEAK (Tumor necrosis

factor-like weak inducer of apoptosis) was found to be in close correlation with inflammation and hemorrhagic severity, and represent a potential biomarker for predicting clinical outcome after aSAH.

Oncostatin-M (OSM) is a member of the glycoprotein 130 (or IL-6/LIFR—leukaemia inhibitory factor receptor) cytokine family. The role of OSM has already been specified in joint, skin, lung, and vascular homeostasis and disease. The cytokines including OSM are essential components of the inflammatory process that provide survival signals to neurons. Within the CNS, the major cellular sources of OSM are astrocytes, neurons, microglia and infiltrating immune cells. Several studies reported that it has a protective effect in many central nervous system diseases such as demyelinating diseases, ischemic stroke, and spinal cord injury. In. contrast, at the level of the blood-brain-barrier (BBB) a pro-inflammatory readout of OSM have been observed Presumably, there is an interplay between TNFSF14/LIGHT and OSM in cellular interaction between intra-arterial smooth muscle cells and peripheral blood mononuclear cells (predominantly T cell) during chronic neuroinflammation, as it was found in airway inflammation.

Fatty acid–binding protein 3 (FABP3), also known as heart-type FABP (H-FABP), is a low-molecular-weight (15 kDa) lipid-binding protein, highly expressed in the cytoplasm and released rapidly from damaged cells into the circulation. The serum level FABP3 were elevated within 3 hours after stroke or myocardial ischemia, suggesting its role as a potential indicator of cellular injury. Recently have been shown that aSAH patients with clinical sign of cerebral vasospasm or unfavorable neurological outcome at 30 days have higher FABP3 levels.

Chemokines are known to play an important role in atherogenesis and vascular inflammation. The novel CXC-chemokine ligand 16 (CXCL16) is both an interferon (IFN) γ regulated chemokine and a scavenger receptor that is up-regulated in macrophages. Ueland et al. showed that the increase in plasma levels of CXCL16 during the first days after acute ischemic stroke is associated with an adverse outcome. Furthermore, increased circulating levels have also been reported in patients with acute myocardial infarction (AMI) and related to adverse clinical outcomes, suggesting its key role in the pathogenesis of vascular diseases.

2. Aims

The aim of the present thesis is to test if neuroinflammatory biomarkers released by pathophysiological processes during aneurysmal subarachnoid bleeding have a potential to predict outcome or disease progression.

Specific aims:

1. Our first aim was to prove evidence that the systemic concentration of LIGHT, OSM, FABP3 and CXCL16 measured within 24 hours in patients with aSAH differ from age matched healthy controls.

2. Secondly, we also intended to investigate the 30-day prognostic value of serum levels of LIGHT, OSM, FABP3 and CXCL16 in patients suffering from aSAH.

3. Thirdly, we aimed to explore the association between the serum concentration of such molecules and DCI.

4. Finally, we intended to investigate if there is any association between the measured biomarkers.

3. Methods

In this prospective, observational study, consecutive aSAH patients were enrolled at Department of Neurosurgery, University of Pecs, between October 2018 and September 2020. Inclusion criteria for the studies were:

- age >18 years;
- clinical history of aneurysmal SAH with an aneurysm identified on CT angiography or digital subtraction angiography within 24 h after symptom onset; and
- informed consent obtained from the patient or legal representative.

Exclusion criteria were:

- rebleeding with a minimum 3-point drop in GCS or a 4-point increase in NIHSS scale after admission and before endovascular treatment;

- malignant or autoimmune disorder;
- active infectious diseases (symptoms of infection with fever, elevated C-reactive protein or procalcitonin, and a positive diagnostic test such as chest X-ray or urine test;
- estimated glomerular filtration rate, eGFR <50 and/or creatinine >120 μmol/L at two distinct measurements;
- evidence of concomitant coronary syndrome (if all of the followings are met: troponin-I value >14 ng/L; typical clinical symptoms; characteristic ECG changes);
- unavailable biomarker measurements;
- refusal of participation; and
- previous modified Rankin Scale (mRS)>2.
- patients receiving drugs or treatments that affect immune functions were also excluded.

Age-matched healthy controls were recruited by the Department of Immunology and Biotechnology, where all biomarkers were measured. The disease severity in aSAH patients was assessed on hospital admission using the World Federation of Neurosurgeons scale (WFNS) and modified Fisher score system (mFisher). Demography, clinical and laboratory data, as well as medical history, were recorded. Other relevant variables such as need for mechanical ventilation, decompressive craniotomy, or ventricular drainage were also explored.

The study protocol was approved by the Local Ethics Committee at University of Pecs, Faculty of Medicine, and informed consent was obtained from each patient according to the "good clinical practice" (GCP) guidelines (35403-2/2017/EKU).

3.1. Outcome

We aimed to evaluate the association of biomarkers with

(1) unfavorable (mRS score 3-6) vs. favorable (mRS score 0-2) outcome at Day 30 as the primary endpoint
(2) DCI as secondary endpoint,
(3) Day 30 mortality as tertiary endpoint to form surviving (mRS score<6) and non-surviving (mRS score=6) patient groups.

Patients were classified as having DCI if

(1) presenting with a change in level of consciousness (a decrease of at least 2 points in the GCS or an increase of more than 2 points in the National Institute of Health Stroke Scale (NIHSS)) or development of new focal deficit lasting for at least 1 h and not explained by other factors;
(2) having a new Diffusion Weighted Imaging (DWI) lesion on MRI obtained after the suspected DCI;
(3) all previously mentioned criteria are met.

The definition of DCI used in the study was based on an AHA recommendation published in 2010.For follow-up, we used structure telephone interviews performed by 1 doctor, blinded to clinical information.

3.2. Sample collection

Arterial blood samples were drawn from each patient on admission within 24 h after the onset of symptoms, immediately before neurointervention. The samples were immediately centrifuged at 400 r/min for 15 min. The supernatant was stored at −80°C until analysis. LIGHT/TNFSF14, OSM, FABP3 and CXCL16 concentrations were determined by using MILLIPLEX MAP Human Cardiovascular Disease Magnetic Bead Panel 1—Cardiovascular Disease Multiplex Assay (Merck KGaA, Darmstadt, Germany). Troponin-I was also measured by the same assay to exclude ongoing coronary syndrome. All samples were processed by the same technicians using the same equipment, blinded to all clinical data. The detection limits for the assay were 43 pg/ml for troponin-I, 1.6 pg/ml for LIGHT/TNFSF14, 0.6 pg/ml for OSM, 23.7 pg/mL for FABP3, and 11.9 pg/mL for CXCL-16.

3.3. Statistical analysis

Data were evaluated using SPSS (version 11.5; IBM, Armonk, NY, USA). The Kolmogorov-Smirnov test was applied to check for normality. To analyse demographic and clinical factors, the chi-square test was used for categorical data while the Student *t* test was applied to continuous datasets. Non-normally distributed data were presented as median and interquartile range and were compared with the use of Mann-Whitney test. The cutoff value with the best sensitivity and specificity of LIGHT/TNFSF14, OSM, FABP3 and CXCL16

were measured (n=60) to determine 30-day survival, and delayed cerebral ischemia was calculated by receiver operator curve (ROC) analysis. Correlation analysis was performed by calculating Spearman's correlation coefficient (rho). To explore the independent predictors of survival, a binary logistic regression was used. A p-value <0.05 was considered statistically significant.

4. Results

4.1. Patients characteristics

During the study period, 98 patients were initially assessed. 38 patients were excluded because of the reasons explained in **Figure 1.** Eventually, 60 aSAH patients were included into this study. The distribution of the eligible aSAH patient-group was 28 (46.7%) males and 32 (53.3%) females with an average age of 58 ± 11 years (range: 27-80 years). Intergroup differences were not statistically significant in gender and age compared between the patients and the 21 healthy controls. In the eligible aSAH patient-group, the admission median WFNS score was 2 (IQR: 1-4), and the admission median Fisher score was 3 (IQR: 3-4). The localization distribution of the aneurysm was the following: 10 (16.7%) posterior communicating artery; 10 (16.4%) internal carotid artery; 17 (28.3%) anterior communicating artery; 9 (15%) middle cerebral artery; 7 (11.7%) anterior cerebral artery; and 7 (11.7%) aneurysms were located in the vertebral and basilar artery. In the study-group, 24 (40%) patients required ventricular drainage; 31 (51.7%) required mechanical ventilation during hospitalization and DCI developed in 16 (26.7%) cases. The mean admission serum C-reactive protein (CRP) level was 49.5±63 mg/L; the admission mean neutrophil-lymphocyte ratio (NLR) was 7.7±5, and the mean admission serum creatinine level was 63.5±21.6 μmol/L.

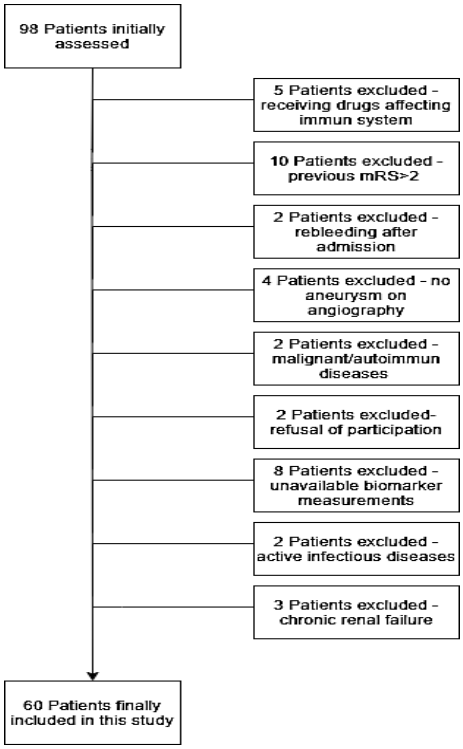

Figure 1 *Flow chart illustrating excluded and included patients with aneurysmal subarachnoid hemorrhage*

4.2. Biomarker levels in patients and controls

The serum FABP3, TNFSF14/LIGHT and OSM levels measured within 24 h after symptom onset were significantly elevated in the serum of the patients compared with healthy controls (median of FABP3 3325 pg/mL, IQR: 1548-5297 vs 1373 pg/mL, 811-2498, p=0.003; median of TNFSF14/LIGHT: 18.1 pg/mL, IQR: 7.1–46.7 vs. 7.1 pg/mL, 7.1–7.3 and median of OSM: 10.3 pg/mL, 7.5–16.4 vs. 2.8 pg/mL, 2.8–4.9, $p < 0.001$, respectively). The serum CXCL-16 levels showed no differences between patients and their age-matched controls (462 pg/mL, 360-563 vs. 431 pg/mL, 307-498, p=0.242).

4.3. Subgroup analysis for patients with favorable and unfavorable outcome

The results of sub-population analysis for the markers showed that the patients with unfavorable outcome revealed higher FABP3 and CXCL-16 concentrations (p=0.003, p< 0.001, respectively), **Table 1.** Meanwhile no significant difference in serum TNFSF14/LIGHT level was observed between the two groups (favorable, median: 20.2 pg/ml IQR (7–50) vs. unfavorable, 16.6 pg/ml IQR (7–46), p = 0.652). However, when patients were dichotomized according to mRS 0–3 as favorable vs. mRS 4–6 as unfavorable outcome,

a statistically significant difference emerged in the serum level of TNFSF14/LIGHT (favorable, median 24 pg/ml (7–59) vs. unfavorable 10.2 pg/ml (7.1–28), p = 0.048).

	Favorable (n=34)	Unfavorable(n=26)	p-value
Female	20 (58.8%)	12 (46.2%)	0.330
Age (y)	56.2±11.4	60.4±10.4	0.189
Diabetes mellitus	3 (9.1%)	6 (23.1%)	0.138
WFNS grade on admission	2 (1-2)	4 (3-5)	<0.001*
mFisher score on admission	3 (2-3)	4 (3-4)	0.002*
Ventricular drainage	7 (20.6%)	17 (65.4%)	<0.001*
Mechanical ventilation	8 (23.5%)	23 (88.5%)	<0.001*
Decompressive craniectomy	2 (5.9%)	5 (19.2%)	0.110
Creatinine (mmol/L)	61 (46-78)	61 (47-66)	0.730
CRP (mg/L)	7.3 (3-48)	59 (34-96)	<0.001*
NLR	6.5±5	9.2±5	<0.001*
PLR	169±66	217±135	0.135
Infection during hospitalization	5 (14.7%)	14 (53.9%)	0.005
Delayed cerebral ischemia	5 (14.7%)	11 (42.3%)	0.017
Endocrine disorder during hospitalization	8 (24.2%)	5 (19.2%)	0.645
serum TNFSF14 (pg/mL)	20.2 (7-50)	16.6 (7-46)	<0.652
serum CXCL-16 (pg/mL)	384 (313-502)	498 (456-623)	<0.001*
serum FABP3 (pg/mL)	2133 (1053-4567)	3773 (3295-13116)	0.003*
serum OSM (pg/mL)	9.68(7-12)	11.9 (8-19)	0.167

Table 1. *Comparison of clinical and biochemical characteristics between patients with favorable (mRS score 0-2) and unfavorable (mRS 3-6) outcome at Day 30 follow-up with aneurysmal subarachnoid hemorrhage. The categorical variables are presented as frequency and percentage, and the continuous variables are presented as mean ± standard deviation or median (percentile 25–75). WFNS indicates World Federation of Neurological Surgeons; mFisher, modified Fisher; CRP, C-reactive protein; NLR, neutrophil-lymphocyte ratio; PLR, platelet-lymphocyte ratio; CXCL-16, Chemokine (C-X-C motif) ligand 16; FABP3, Fatty-acid-binding protein 3.*

4.4. Independent predictors of unfavorable outcome

The area under the curve (AUC) for serum CXCL-16 levels as a predictor of unfavorable outcome at Day 30 was 0.747 (95% CI =0.622-0.871; p< 0.001). Based on ROC analysis, serum CXCL-16 level > 446.7 ng/L predicted Day 30 unfavorable outcome of patients with a sensitivity of 80.8% and a specificity of 61.8%. Another ROC analysis of serum FABP3 as a predictor of unfavorable outcome on Day 30 revealed a cut-off level of 3149.3 pg/mL (AUC of 0.724, 95% CI= 0.591-0.857; p=0.003) with an 80.8% sensitivity and

67.6% specificity. TNFSF14 was not proven to be an independent predictor of unfavourable outcome, just for mortality alone.

Table 1. showed variables associated with unfavorable outcome at Day 30 included mFisher score, WFNS score, serum CRP level, neutrophil-lymphocytes ratio, serum FABP3 level> 3149.3 pg/mL and CXCL-16 levels > 446.7 pg/mL. When the above-mentioned variables were used in a binary logistic regression model, in addition to the most common determinants for poor outcome (modified Fisher score and WFNS score), serum CXCL-16 level > 446.7 pg/mL was found as an independent predictor for unfavorable Day 30 outcome. **Table 2**.

	unfavorable outcome at Day 30			
Variables	B	**Odds ratio**	**95% CI**	**p-value**
CXCL-16 level> cutoff	-0.264	5.980	0.017-0.638	0.014*
creatinine	0.048	2.685	0.991-1.112	0.101
mFisher score	0.454	0.495	0.445-5.573	0.482
WFNS score	0.439	1.127	0.690-3.489	0.288
CRP	0.010	1.118	0.992-1.028	0.290
FABP3 level> cutoff	-1.006	0.933	0.047-2.816	0.334

Table 2. *Binary logistic regression analysis for variables associated with unfavorable outcome (mRS>2) on day 30 in patients with aneurysmal subarachnoid hemorrhage. CRP, C-reactive protein; mFisher score, modified Fisher score; WFNS, World Federation of Neurosurgical Societies score; CXCL-16, Chemokine (C-X-C motif) ligand 16; cutoff level for CXCL-16: 446.7 pg/mL; FABP3, Fatty-acid binding protein-3, cutoff level for FABP3: 3149.3 pg/mL;* ∗ *indicates significant correlation (p<0.05)*

CXCL-16 and FABP3 were significantly correlated with a number of factors in univariate analysis (as shown in **Table 3.**). Variables associated with FABP3 included mFisher score, WFNS score, serum creatinine, serum CRP, platelet count and NLR both correlated with mechanical ventilation, ventricular drainage and mean GCS on Day 4-10.

Parameter	CXCL-16	FABP3
mFisher score	0.253	0.537**
WFNS	0.200	0.538**
s-creatinine (mg/dl)	-0.268*	-0.308*
C-reactive protein (mg/L)	0.116	0.343*
platelet	-0.101	-0.428**
NLR	0.217	0.295*
GCS (mean at Day 4-10)	-0.290*	-0.439**
FABP3	0.426**	N/A
CXCL-16	N/A	0.426**
Delayed cerebral ischemia	0.174	0.104

Table 3. *Variables associated with CXCL-16 and FABP3 in cross-sectional univariate analysis. Values are Spearman correlation coefficients (rho). ⁎P < 0.05; ⁎⁎P < 0.001. CRP, C-reactive protein; mFisher score, modified Fisher score; WFNS, World Federation of Neurosurgical Societies score; CXCL-16, Chemokine (C-X-C motif) ligand 16; FABP3, Fatty-acid binding protein; NLR, neutrophil-lymphocyte ratio; GCS, Glasgow coma scale*

4.5. Subgroup analysis between nonsurviving and surviving patients

Furthermore, we compared demographic and clinical parameters between nonsurviving (n = 9) and surviving (n = 51) patients **(shown in Table 4.)**. A significantly higher serum concentration of LIGHT/TNFSF14 was observed in survivors compared with nonsurvivors (median: 22.5, IQR: 7–50 vs. 7.14, 7–7.14, p = 0.011) and a significant correlation was observed between LIGHT/TNFSF14 and OSM in the sera of surviving patients (p < 0.001) **(Figure 2.).**

	Non-survivors (n=9)	Survivors (n=51)	p-value
Female	5 (55.6%)	27 (52.9%)	0.885
Age (y)	54.8±6.7	58.6±11.6	0.325
Diabetes	1 (11.1%)	8 (16%)	0.707
WFNS grade on admission	5 (3-5)	2 (1-3)	0.007*
mFisher score on admission	4 (4)	3 (2-4)	0.005*
Ventricular drainage	6 (66.7%)	18 (35.3%)	0.077
Decompressive craniectomy	1 (11.1%)	6 (11.8%)	0.955
Creatinine (mmol/L)	71.4±30	62±20	0.363
CRP (mg/L)	92±94	41±52	0.052
NLR	7.1±5	7.8±5	0.874
PLR	202±102	188±106	0.392
Infection during hospitalization	4 (50%)	15 (29.4%)	0.390
Delayed cerebral ischemia	4 (44.4%)	12 (24%)	0.191
Endocrine disorder during hospitalization	1 (11.1%)	12 (24%)	0.645
LIGHT/TNFSF14 (pg/mL)	7.14 (7)	22.5 (7-50)	0.011*
Oncostatin-M (pg/mL)	10.3 (3.9-12.6)	10.3 (8.3-17.3)	0.419
FABP3 (pg/mL)	3354 (3224-3894)	3075 (1496-5929	0.928
CXCL-16 (pg/mL)	162 (126-315)	222 (171-275)	0.702

Table 4. *Comparison of clinical and biochemical characteristics between non-survivors and survivors with aneurysmal subarachnoid hemorrhage The categorical variables are presented as frequency and percentage, and the continuous variables are presented as mean ± standard deviation or median (percentile 25–75). WFNS indicates World Federation of Neurological Surgeons; mFisher, modified Fisher; CRP, C-reactive protein; NLR, neutrophil-lymphocyte ratio; PLR, platelet-lymphocyte ratio; LIGHT/TNFSF14, Tumor necrosis factor superfamily 14; ∗ indicates significant correlation (p<0.05).*

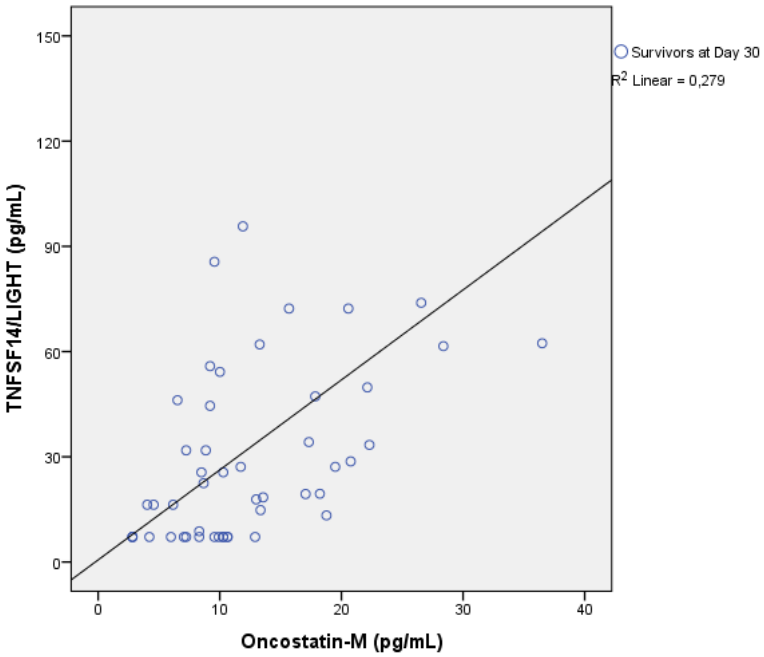

Figure 2. *Scatter-dot graph showing the positive correlation between serum LIGHT/TNFSF14 and Oncostatin-M levels measured 24 hours after the onset of symptoms in the survivor subgroup in patients with aneurysmal subarachnoid hemorhage (p=0.001, 2-tailed).*

4.6. Independent predictor of mortality

The serum level of LIGHT/TNFSF14 within 24 h and mFisher score assessed on admission were independently associated with Day 30 survival, whereas age, gender, DCI, and in-hospital infection rate were not **(Table 5.)**. The serum LIGHT/TNFSF14 with a cutoff value of >7.95pg/ml predicted Day 30 survival with a sensitivity of 71% and specificity of 78% (area: 0.763; 95% CI: 0.604–0.921, $p = 0.013$). When LIGHT/TNFSF14 level was combined with other variables such as mFisher score or WFNS score, the two-variable ROC analysis explored improved predictive power of mortality of both combination (LIGHT/TNFSF14+mFisher, Area: 0.878, 95% CI: 0.78–0.98;$p<0.001$, and LIGHT/TNFSF14+WFNS, Area: 0.829, 95% CI: 0.72–0.94;$p=0.002$). However, the combination of LIGHT/TNFSF14+presence of DCI and LIGHT/TNFSF14+OSM showed a weaker predictive power (Area: 0.769, 95% CI: 0.61–0.93; $p = 0.011$; Area: 0.758, 95% CI: 0.59–0.93;$p=0.014$, respectively).

30 day survival				
Variables	**B**	**Odds ratio**	**95% CI**	**p-value**
LIGHT/TNFSF	0.067	4.096	1.002-1.141	0.043*
Age	0.097	2.952	0.986-1.231	0.086
gender	0.019	0.000	0.119-8.730	0.986
mFisher score	-2.357	4.358	0.010-0.866	0.037*
DCI	0.570	0.221	0.164-19.035	0.639
Infection	0.158	0.124	0.486-2.825	0.724

Table 5. *Binary logistic regression analysis for variables independently associated with survival (mRS<6) on Day 30 in patients with aneurysmal subarachnoid hemorrhage. DCI, delayed cerebral ischemia; LIGHT/TNFSF14, Tumor necrosis factor superfamily 14; mFisher score, modified Fisher score;* ∗ *indicates significant correlation (p<0.05)*

4.7. Prediction of DCI

Notably, a significantly lower serum level of LIGHT/TNFSF14 was observed within 24 h in patients later developing DCI than in patients without such complication (median: 7, IQR: 7–19 vs. 25.6, (7–51), p = 0.015). In the same comparison, neither CXCL-16 nor FABP3 nor OSM showed a significant correlation with DCI.

A separate analysis was run with DCI as the outcome of interest. Based on binary logistic regression analysis, age (OR: 0.9, 95% CI: 0.823–0.984, *p*=0.02) and C-reactive protein (OR: 1.02, 95% CI: 1.001–1.033,*p*=0.03) proved to be independent predictors of DCI, but not LIGHT/TNFSF14 (*p*=0.06). Interestingly, the ROC analysis of serum LIGHT/TNFSF14 level as a predictor of DCI during hospitalization revealed the same cutoff level of 7.95pg/ml (AUC of 0.702, 95% CI: 0.555–0.849; *p*=0.018) with a 72.7% sensitivity and 62.5% specificity.

5. Discussion

In the current study, we analysed the medical data of 60 aSAH patients and determined the serum LIGHT/TNFSF14, OSM, CXCL-16 and FABP3 concentrations within 24 h after the onset of symptoms. A relationship between these biomarker levels, clinical outcome and survival of patients with aSAH, as well as potential predictive values for DCI, were assessed.

Several mechanisms are responsible for aSAH-related mortality. The systemic inflammatory response syndrome can also influence the overall survival

SAH cause brain injury via glutamate excitotoxicity, leading to an excessive Ca^{2+} influx and can induce an apoptotic cascade. CSF levels of glutamate are significantly higher in patients with severe SAH than those with less severe SAH. CXCL-16 can promote physiological neuroprotective mechanisms that counteract neuronal cell death due to ischemic and excitotoxic insults mediated by glutamate. CXCL-16 acts directly on astrocytes to release soluble factors essential to mediate neuroprotection against excitotoxic damage due to excessive glutamate exposure. In addition, both serum and CSF CXCL-16 levels are significantly elevated in various inflammatory conditions such as bacterial- and viral meningitis, multiple sclerosis and systemic lupus erythematosus. These findings suggest an essential role for CXCL-16 in the regulation of T cell homing to the CNS. Our finding, that elevated serum level of CXCL-16 is an independent predictor of unfavorable outcome may indicate the importance of the thrombo-inflammatory system on the outcome of aSAH. The more severe the neuronal damage, the more pronounced neuroprotective mechanisms are triggered; however, these do not necessarily improve the final outcome due to the extent of irreversible damage.

Recent studies suggest that a high serum level of CXCL-16 is independently related to adverse clinical outcomes both in coronary atherosclerotic heart disease and in acute coronary syndrome. There may be several explanations for these results: (1) CXCL-16 activates CD8+ T cells, leading to apoptosis in the surrounding of an atherosclerotic plaque; (2) CXCL-16 has the ability to direct the migration of activated T lymphocytes to the lesion tissue; (3) where it can promote plaque formation and thrombosis by locally secreting multiple cytokines and matrix metalloproteinases. Both apoptosis and damaged cerebral microvasculature contribute to the pathological processes of subarachnoid hemorrhage; thus, an elevated systemic CXCL-16 level may play a role in SAH-induced tissue damage through these mechanisms. It is known that the CXCL-16 molecule has a membrane-bound and a soluble form with entirely different biological functions. The soluble form is primarily a chemoattractant, while the transmembrane form promotes the adhesion of lymphocytes. Though, the two forms have been suggested to have an opposite function regarding trombo-immflamatory processes. Similar to TNFSF14, which has also been emerged as a novel marker of the outcome in aSAH recently, CXCL-16 is a "Janus" faced molecule exerting both pro- and anti-inflammatory effects. Further investigation is needed to measure the distinct forms of CXCL-16 separately, and determine the exact mechanism behind the opposite functions.

Another result of the study revealed that the serum level of FABP3 was significantly elevated in patients with unfavorable outcome after aSAH. We also found that FABP3 level

was positively correlated with SAH severity scores (WFNS and mFisher), CRP and NLR, as well as the serum level of CXCL-16, while negatively correlated with serum creatinine, platelet count and GCS. In accordance, it has been found that serum level of FABP3 showed significant association with Hunt Hess score and Fisher score in a small cohort of patient with SAH. In a recent study, the CSF level of FABP3 showed significantly higher peak levels in patients with severe SAH than mild SAH patients. A study. showed that serum FABP3 was elevated early in acute ischemic stroke, indicating that it might have the potential to be a rapid marker of brain damage and clinical severity. Importantly, FABP3 is a more sensitive marker for minor brain injury than the commonly used markers such as S100B or NSE. Moreover, it has an excellent ability to differentiate between CT-positive and CT-negative mild TBI patients. FABP3 is also known to be a sensitive marker in acute coronary events and cardiac failure. Therefore, we measured hs-troponin and NT-pro BNP to rule out the cardiac source of FABP3 in our study. FABP3 is predominantly found in neuronal tissue and constitutes 0.01% of the total brain cytosolic protein. Its serum concentration remains increased for days after ischemic stroke; thus, its measurement at 24 hours after the index event in the systemic circulation may reflect the extent of tissue damage in our cohort. Presumably, a tissue damage process like an ischemic insult may produce a high concentration of long-chain fatty acids, which can influence or disrupt cellular processes, such as enzyme functions (Na-K ATPase). FABP3 can bind the long-chain fatty acids accumulating under these pathophysiological circumstances, and it plays a role in removing these fatty acids from the cell and protecting them against its deleterious effects.

We hypothesized that the background of high levels of FABP3 observed in our patients with SAH is similar to those previously observed in other pathological processes like mild traumatic brain injury or ischemic stroke. In our study, we observed a significant positive correlation between CXCL-16 and FABP3. Notably, such association has never been reported in the literature before. Considering that both markers are key players in different pathological mechanisms, their observed correlation in our study deserves further exploration.

At first sight, our results are inconsistent with the current literature discussing the role of the LIGHT/TNFSF14 protein in patients with coronary artery diseases (CAD). Enhanced plasma levels of LIGHT/TNFSF14 was found in unstable angina. and increased LIGHT/TNFSF14 levels were independently associated with the occurrence of cardiovascular events in patients with stable CAD. LIGHT/TNFSF14 is also known to be involved in orchestrating uncontrolled immune response resulting in autoimmunity and tissue injury diseases such as inflammatory bowel disease, asthma, and lung fibrosis In contrast, other

publications suggest a protective role of increased systemic TNFSF14 concentration. Elevated serum levels of LIGHT/TNFSF14 during relapses in multiple sclerosis (MS) indicate that the soluble form of LIGHT/TNFSF14 may act as a compensatory mechanism to decrease the stimulatory signals through herpesvirus entry mediator (HVEM) and thereby limit the inflammation in MS patients during relapse. The decrease in serum levels of LIGHT/TNFSF14 by natalizumab treatment in MS patients suggests that LIGHT/TNFSF14 decreases when inflammation is reduced by disease-modifying treatment. LIGHT/TNFSF14 was found to be significantly reduced in concussed patients when compared with healthy individuals. LIGHT/TNFSF14 engages two cellular signalling receptors, lymphotoxin receptor (LTR) and HVEM, and is inactivated by Decoy receptor-3 (DcR3). Circulating LIGHT/ TNFSF14 is unbound to DcR3. This "free LIGHT/TNFSF14" is the soluble form that retains receptor-binding activity. This form has been suggested to have an opposite function to that of the membrane-bound form and is an inhibitor of T-cell activation. The membrane-bound LIGHT/TNFSF14 seems to have diverse stimulatory effects on the immune system and may also have the potential to induce autoimmunity. Another member of the TNF superfamily, TWEAK, has significantly higher serum levels in patient with poor outcome after SAH. Like LIGHT, two forms of TWEAK are known as the soluble form and the membrane-bound form. The two forms may have different roles in biochemical processes, similar to what we see in case of LIGHT. According to our hypothesis, the ratio of the two form of LIGHT (soluble vs. membrane-bound) at the time of measurement may be decisive in their role after SAH. Taken together, there are several promising theories to explain the low serum level of LIGHT/TNFSF14 in nonsurvivor aSAH patients.

Firstly, it is known that the degradation of LIGHT/TNFSF14 by matrix metalloproteinases (MMPs) contributes to the return to baseline levels of both LIGHT/TNFSF14 and HVEM. Level of several types of MMPs was significantly higher in aSAH cases compared with controls and subarachnoid bleeding significantly upregulates both the expression and activity of matrix metalloproteinase-9 (MMP-9) in blood and CSF. Several experimental models suggest that MMP-9 activity mediates blood-brain barrier breakdown and subsequent vasogenic oedema following SAH. Thus, higher concentrations of MMPs may theoretically reduce LIGHT/TNFSF14 levels in nonsurvivor aSAH patients.

Secondly, the upregulation of the DcR3 may be significant in SAH due to its proinflammatory actions. Its neutralizing effect on LIGHT/TNFSF14 may contribute to the detrimental outcome after aSAH. Several studies revealed increased serum DcR3 levels in some chronic inflammatory conditions, such as sepsis, acute respiratory distress syndrome,

and cardiovascular disease. Interleukin-6 upregulates DcR3 expression. On the other hand, elevated serum concentration of IL-6 was detected in patients with unfavorable outcome of SAH. Therefore, it seems reasonable to presume that upregulated DcR3 may decrease the measurable level of LIGHT/ TNFSF14 in the sera of nonsurvivor aSAH patients.

Thirdly, higher subacute level of LIGHT/TNFSF14 in the sera of surviving patients highlights a protective role of LIGHT/TNFSF14 in SAH-related neuroinflammation. Some evidence stated that TNFSF14 promoted recovery from intestinal inflammation in mice. They observed a more severe disease phenotype in both colitis models in the absence of LIGHT/TNFSF14 expression, or when LIGHT/TNFSF14 interaction with one of its receptors (HVEM and LTβR) was blocked. In a mouse model of colitis, LIGHT/TNFSF14 played an important role in protection from inflammation. In addition, more severe disease pathogenesis was observed in LIGHT/TNFSF14-deficient mice.

Importantly, OSM concentration showed a strong positive correlation with the level of LIGHT/TNFSF14 in the sera of surviving patients. It has been proposed that OSM may induce JAK2/STAT3-mediated neuroprotection during ischemic stroke. The neuroprotective effect induced by STAT3 activation has been reported by many authors. A recent review stated indicated that OSM significantly attenuated excitotoxic cell death in both in vitro and vivo. Based on these findings, OSM is considered as a novel neuroprotective cytokine. It was found that the production of OSM continually increased from 12 to 72 h in the brain when using a middle cerebral artery occlusion (MCAO) rat stroke model. Furthermore, treatment with OSM significantly improved the neurofunctional recovery, while the expression of inflammatory mediators was reduced. Mikami et al also showed that LIGHT/TNFSF14 induced OSM production by bronchial epithelial cells. In summary, the observed association between LIGHT/TNFSF14 and OSM with survival is a novelty. Accordingly, we may hypothesize that coexpression of the two ligands is required for suppression of the SAH-related inflammatory process resulting in a better functional outcome in SAH patients.

In our study, we also found significantly lower serum LIGHT/ TNFSF14 level in patients with DCI compared with those without, but this was not proved to be an independent predictor of DCI. Importantly, no correlation was found between serum levels of CXCL-16 and FABP3 and the incidence of delayed cerebral ischemia in our cohort. It is known that DCI develops between days 4 and 12 after subarachnoid hemorrhage. A variety of mechanisms are implicated in symptomatic cerebral vasospasm and DCI after SAH. Recent studies have provided evidence that cerebral vasospasm is not the only contributor to DCI, and additional mechanisms may play equally important roles. We previously described that

LIGHT/TNFSF14 and CXCL-16 has both pro- and anti-inflammatory properties and neuroinflammation has been associated with the development of DCI. Nevertheless, a recently published study found no association between DCI and inflammatory molecules such as IL-6, IL-8, IL-10, ICAM-1, VCAM-1, and IFNγ[i]. Microthrombi also contribute to the development of DCI. The absence of LIGHT/ TNFSF14 from the cell surface of platelets causes rapid platelet aggregation. Another study concluded that platelet-associated LIGHT/TNFSF14 is involved in adhesion of platelets to the endothelium, while soluble LIGHT/TNFSF14 induces a proinflammatory state in vascular endothelial cells contributing to thrombus formation. Presumably, the lack of elevation of LIGHT/TNFSF14 in nonsurvivors may suggest its potential protective effect independently from complications such as development of DCI. In our study, biomarker sampling occurred 24 hours after the onset of symptoms; thus, their systemic concentration may not have been informative regarding late complications such as DCI. Alternatively, the impact of systemic LIGHT/TNFSF14, CXCL-16 or FABP3 levels on the outcome in aSAH is independent of the presence of DCI at all. However, multiple sampling is recommended in patients with aSAH to explore the kinetics of these markers and their potential link to the development of DCI.

6. NOVEL FINDINGS AND CONCLUSION

Ad Aim I.

The serum FABP, LIGHT/ TNFSF14 and OSM levels were elevated by aSAH patients compared with healthy controls; but not CXCL-16.

Ad Aim 2.

Lower serum CXCL-16 and FABP3 levels measuring right after admission predicted 30-day favorable clinical outcome. Moreover, a positive correlation emerged between CXCL-16 and FABP levels in our cohort.

Ad Aim 3.

The elevation of the serum levels of LIGHT/TNFSF14 measured at admission served as an independent predictor of survival after aSAH. In addition, a positive correlation was also found between LIGHT/TNFSF14 and OSM levels in survivals.

Ad Aim 4.

An association have been explored between lower serum LIGHT/ TNFSF14 levels and development of DCI, but not regarding the other examined molecules.

In summary, the protective role of higher serum LIGHT/TNFSF14 levels emerged from this cohort, while the elevation of CXCL-16 and FABP3 in the serum are independently related to adverse clinical outcomes. The pro- and anti-inflammatory properties of these biomarkers showing the Janus face of these molecules have been reported under different clinical conditions. However, the exact pathophysical mechanism behind the opposite effects needs to be further investigated.

Despite numerous limitations of this prospective study, our preliminary data may generate further translational research contributing to a better understanding of the complex inflammatory response after aSAH. The potential beneficial role of the CXCL-16, FABP3, LIGHT/TNFSF14 and OSM system should be explored in future SAH trials providing potential therapeutic targets and more precise predictive value regarding the outcome and severity of SAH. Since DCI is a multifactorial and extremely serious complication after SAH, the role of inflammatory molecules needs to be more thoroughly examined in this process. Therefore, larger prospective studies involving a broad spectrum of inflammatory molecules measuring not only the molecules found in the serum, but the membrane-bound form too, in patients with aSAH, would justify clarification of those clinical utility.

7. LIST OF PUBLICATION

7.1. Publications related to the thesis

1. **Schranz D,** Molnar T, Erdo-Bonyar S, Simon D, Berki T, Nagy C, Czeiter E, Buki A, Lenzser G, Csecsei P. Increased level of LIGHT/TNFSF14 is associated with survival in aneurysmal subarachnoid hemorrhage. Acta Neurol Scand. 2021 May;143(5):530-537. doi: 10.1111/ane.13394.
IF: 3,209

2. **Schranz D**, Molnar T, Erdo-Bonyar S, Simon D, Berki T, Zavori L, Szolics A, Buki A, Lenzser G, Csecsei P. Fatty Acid-Binding Protein 3 and CXC-Chemokine Ligand 16 are Associated with Unfavorable Outcome in Aneurysmal Subarachnoid Hemorrhage. J Stroke Cerebrovasc Dis. 2021 Nov;30(11):106068. doi: 10.1016/j.jstrokecerebrovasdis.2021.106068.
IF: 2,126
Cumulative impact factor related to the thesis: **5,335**

7.2. Other publications

1. Horváth RA, Sütő Z, Cséke B, **Schranz D**, Darnai G, Kovács N, Janszky I, Janszky J. Epilepsy is overrepresented among young people who died from COVID-19: Analysis of nationwide mortality data in Hungary. Seizure. 2022 Jan;94:136-141. doi: 10.1016/j.seizure.2021.11.013.
IF: 3.184

2. Molnar T, Varnai R, **Schranz D**, Zavori L, Peterfi Z, Sipos D, Tőkés-Füzesi M, Illes Z, Buki A, Csecsei P. Severe Fatigue and Memory Impairment Are Associated with Lower Serum Level of Anti-SARS-CoV-2 Antibodies in Patients with Post-COVID Symptoms. J Clin Med. 2021 Sep 23;10(19):4337. doi: 10.3390/jcm10194337.
IF: 4.202

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