DOCTORAL (Ph.D.) THESIS

Identification of novel predictive biomarkers in patients with aneurysmal subarachnoid hemorrhage

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1.1. ABBREVIATIONS

Acomm	anterior communitating artery
AI	artifical intelligance
AMI	acute myocardial infarction
apoE	apolipoprotein E
aSAH	aneurysmal subarachnoid hemorrhage
ARDS	acute respiratory distress syndrome
AUC	area under the curve
AVM	arteriovenous malformations
BBB	blood-brain-barrier
CAD	coronary artery disease
CNS	central nervous system
CSF	cerebral spinal fluid
CT	computer tomography
CRP	C-reactiv protein
CV	cerebral vasospasm
CXCL-16	C-X-C motif chemokine ligand 16
DCI	delayed cerebral ischemia
DcR3	Decoy receptor-3
DSA	digital substraction angiography
DWI	diffusion wheighted imaging
ECG	electrocardiogramm
EEG	electroencephalogramm
EVD	external ventricular drain
FABP3	fatty acid–binding protein 3
GCP	good clinical practice
GCS	Glasgow Coma Scale
GFAP	glial fibrillary acid protein
HaHs	Hunt and Hass scale
H-FABP	heart type fatty acid binding protein
HVEM	herpesvirus entry mediator
ICH	intracerebral hemorrhage

ICAM-1	intracellular adhesion molecule-1
ICP	intracranial pressure
IFN	interferon
IL-1	interleukine-1
IQR	interquartile range
ISAT	International Subarachnoid Aneurysm Trial
kDA	kilodalton
LTR	lymphotoxin receptor
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
MCP-1	monocyte chemoattractant protein-1
mFISHER	modified Fisher score system
MMP-9	matrix metalloproteinase-9
MRI	magnetic resonance imaging
mRS	modified Rankin scale
MS	multiple sclerosis
NIHSS	National Institute of Health Stroke Scale
NLR	neutrophile-lymphocyte ratio
NSE	neuron-specific enolase
OSM	oncostatin-M
Pcomm	posterior communicating artery
PLR	platelet-lymphocyte ratio
PRES	posterior revesible encephalopathy syndrome
RCVS	reversible cerebral vasoconstriction syndrome
ROC	receiver operator curve
TBI	traumatic brain injury
TNF-α	tumor necrosis factor alpha
TNSF	tumor necrosis superfamily
TNSF-14	tumor necrosis factor superfamily-14
tPA	tissue plasminogen activator
TWEAK	tumor necrosis factor-like weak inducer of apoptosis
SAH	subarachnoid hemorrhage
SD	spreading depolarization
VCAM-1	vascular cell adhesion protein 1

VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
WFNS	World Federation of Neurosurgeons Scale

2. INTRODUCTION

2.1. Definition and epidemiology of aneurysmal subarachnoid hemorrhage

The two major categories of stroke (hemorrhage and ischemia) are 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 [1].

The incidence of aneurysmal SAH (aSAH) varies widely by geographic region. The overall incidence of sSAH is approximately 9/100 000 person/years. Rates are higher in Japan (22,7/100 000) and Finland (19,7/100 000), while Hungary belongs with approximately 10/100 000 person /year (about 1000 case in a year) to the average. 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.[2]

2.2. Etiology, pathogenesis and clinical manifestation of aSAH

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%). Other causes include arteriovenous malformations (AVM) (4-5%), fistulae, vasculitises, intracranial arterial dissections, amyloid angiopathy, cerebral venous sinus thrombosis, tumors, bleeding diatheses, and illicit drug use (especially cocaine and amphetamines).

The prevalence of intracranial saccular aneurysms by radiographic and autopsy series is estimated to be 3.2 percent in a population without comorbidity [3]. Most 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[4].

Saccular aneurysms are responsible for most SAHs, although fusiform and mycotic aneurysms can be identified in selected patients. Intracranial saccular aneurysms are acquired lesions, not congenital.

The pathogenesis of saccular aneurysm formation is multifactorial. Hemodynamic stress causes excessive wear, tear and breakdown of the internal elastic lamina. Turbulent blood flow produces vibrations that may coincide with the resonant frequency of the vessel wall, resulting in structural fatigue. Lack of elastic lamina was a common feature of both ruptured and unruptured aneurysms.

Hypertension, cigarette smoking, and connective tissue diseases such as Ehlers-Danlos syndrome, autosomal dominant polycystic kidney disease probably play a contributory rather than causal role in this process of aneurysm formation. There is some evidence that inflammation plays a pivotal role in the pathogenesis and growth of intracranial aneurysms [5,6].

It is believed that most intracranial aneurysms develop over a short period of hours, days, or weeks, attaining a size allowed by the elasticity limits of the aneurysmal wall; at this point, the aneurysm either ruptures or undergoes stabilization and hardening [7].

Most intracranial aneurysms are asymptomatic unless they rupture, and so they are usually found either incidentally or when a patient presents with SAH. In some cases unruptured aneurysm can cause neurologic symptoms. A pupil-involving third nerve palsy is often cited as a finding of an expanding but unruptured aneurysm of the posterior communicating artery or superior cerebellar artery, which is located close to where the third nerve exits the brainstem[8].

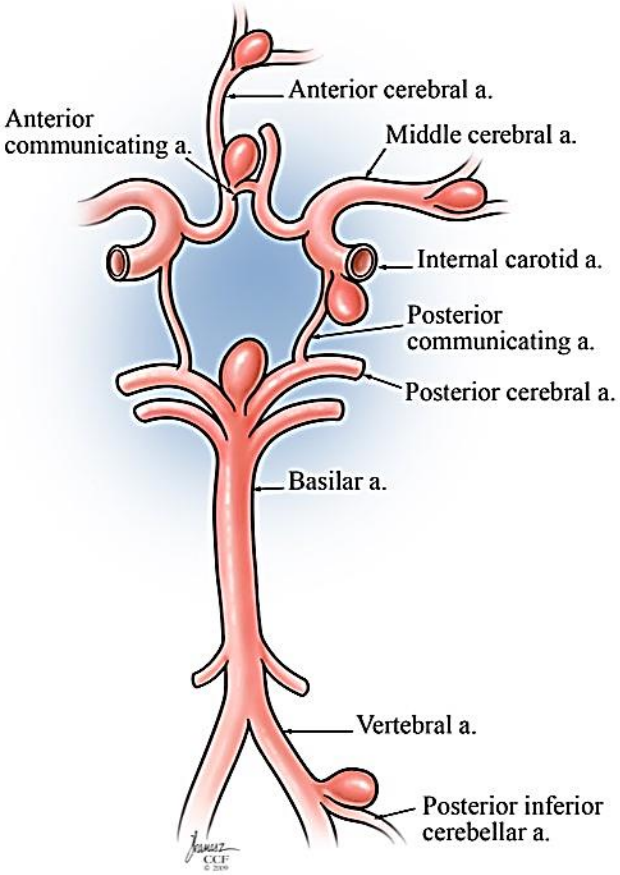

Figure 2.1. *Anatomy of the circle of Willis*

In a serious subarachnoid bleeding even 150 ml bleed is able to escape into the subarachnoid space, more volume is incompatible with life because of the Monroe-Kelly theory. The bleeding stops when the intracranial pressure equals with the intraarterial pressure and additional blood clot formation happens. A few hours later polymorphonuclear leukocytes, lymphocytes and macrophages migrate into the cerebral spinal fluid (CSF).

The classic presentation of patients with aneurysmal SAH is a sudden-onset, severe headache typically described as the "worst headache of my life". Some patients report a history of a sudden and severe headache (the sentinel headache) that precedes a major SAH, occurring days to weeks prior to aneurysm rupture. Sentinel headache may represent either a minor hemorrhage (a "warning leak") or physical changes within the aneurysm wall (e.g., acute dissection, thrombosis, or expansion)[9].

In addition to headache, common associated symptoms of SAH 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 most common neurological signs are shown in Table 1.

In the medical terminology a severe headache of sudden onset is referred as a thunderclap headache. It can be due to multiple possible causes, not only SAH, but other intracranial hemorrhage, reversible cerebral vasoconstriction syndrome (RCVS), posterior reversible encephalopathy syndrome (PRES), hypertensive crisis, cerebral venous sinus thrombosis, cervical artery dissection, or ischemic stroke can be the underlying pathology.

Findings	Likely cause
Third nerve palsy	Usually posterior communicating aneurysm; also posterior cerebral artery and superior cerebellar artery aneurysms
Sixth nerve palsy	Elevated intracranial pressure (false localizing sign)
Combination of hemiparesis and aphasia or visuospatial neglect	Middle cerebral artery aneurysm, thick subarachnoid clots, or parenchymal hematomas
Bilateral leg weakness and abulia	Anterior communicating artery aneurysm
Ophthalmoplegia	Internal carotid artery aneurysm impinging upon the cavernous sinus
Unilateral visual loss or bitemporal hemianopia	Internal carotid artery aneurysm compressing optic nerve or optic chiasm
Impaired level of consciousness and impaired upward gaze	Pressure on the dorsal midbrain due to hydrocephalus
Brainstem signs	Brainstem compression by basilar artery aneurysm
Neck stiffness	Meningeal irritation by the presence of subarachnoid blood
Retinal and subhyaloid hemorrhages	Sudden increase of intracranial pressure
Preretinal hemorrhages (Terson syndrome)	Vitreous hemorrhage due to severe elevations of intracranial pressure

Table 2.1. *Focal physical findings in patients with subarachnoid hemorrhage*

There is an ongoing debate about the topic whether incidentally found intracranial aneurysms should be treated or not. First of all an endovascular expert team have to make indication of the surgery after analysing the anatomical characteristics of the aneurysm, comorbidities of patients, and the overall bleeding risk. What is more important, that the patient

should be well informed about cost/benefit ratio of the procedure. At the end, the patient have to make the decision.

2.3. Standard diagnostic approach, and treatment of SAH

The cornerstone of SAH diagnosis is the noncontrast head computer tomography (CT) scan. The sensitivity of modern head CT for detecting SAH is highest in the first six hours after SAH (nearly 100 percent when interpreted by expert reviewers), and then progressively declines over time to approximately 58 percent at day 5 [10]. The sensitivity of head CT may be reduced with low-volume bleeds. If there is a strong suspicion of SAH despite a normal head CT lumbar puncture is mandatory. The classic lumbar puncture findings of SAH are an elevated opening pressure, an elevated red blood cell (RBC) count that does not diminish from CSF tube 1 to tube 4, and xanthochromia.

Differentiation between arteficial bleeding and CSF blood product can be made by sedimentation. In the case of arteficial bleeding RBCs are going to sediment, while in case of SAH they will not.

Once a diagnosis of SAH has been made, the source of the hemorrhage must be determined with angiographic studies.

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 Substraction Angiography (DSA) is the prefered next diagnostic modality, since we can analyize vascular malformations under higher resolution and at the same time we can iniciatate endovascular treatment if the bleeding source has been found. DSA identifies vascular pathology in about additional 13% of patients with CTA-negative SAH. Aneurysms or pseudoaneurysms are identified in an additional 4% of patients by repeat DSA following an initially negative DSA [11].

The main goal of the initial care of patients with SAH is reversing or stabilizing life-threatening conditions, particularly for comatose patients. Airway protection, cardiovascular function stabilization, and seizure treatment are important initial steps.

After stabilizing vital parameters and making the diagnosis, SAH patients should be transported to high volume center with dedicated neurocritical care unit and expert neurosurgical, neurointerventional and neurointensive care background.

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 [12]. Anatomic considerations, such as size, location, along with other morphological features determine which treatment is most appropriate for the patient.

Surgical management of cerebral aneurysms, in which a clip is placed across the neck of the aneurysm is a safe and effective treatment modality.

Endovascular coiling system was introduced in the early 1990s. The procedure involves inserting a catheter into an artery. Routinely, we puncture the femoral artery, but in selected cases radial artery can be better option. Platinum coils are inserted into the lumen of the aneurysm inducing local thrombus formation around the coils, therethrough obliterating the aneurysmal sac.

New techniques include stent-assisted coiling, balloon-assisted coiling, flow diverters and disruptors, and new embolic material including liquids offer promising results in the treatment of aneurysms, that were not previously considered eligible to endovascular therapy.

In the International Subarachnoid Aneurysm Trial (ISAT), 2143 patients with ruptured intracranial aneurysms were randomly assigned to neurosurgical clipping or endovascular coiling. The patients in this study represented a selected subgroup of patients with low-grade SAH. According to the results endovascular coiling is preferred over clipping, considering higher rate of good funcional outcome even after 7 years follow-up in the endovascular group. However, the occurence of rebleeding is low, but it is more common after endovascular coiling than after neurosurgical clipping [13].

Decisions regarding the timing and choice of therapy for a ruptured intracranial aneurysms are ideally made by a team of experienced clinicians who consider the neurologic grade and clinical status of the patient, the availability of expertise in surgical and endovascular techniques, as well as the anatomic characteristics of the aneurysm.

2.4. Complications after SAH

Medical and neurologic complications are common after SAH and contribute substantially to the overall morbidity and mortality including rebleeding, vasospasm and delayed cerebral ischemia (DCI), hydrocephalus, increased intracranial pressure, seizures, and cardiac complications.

First off all rebleeding is associated with high mortality. Aneurysm treatment via endovascular coiling or neurosurgical clipping is the only effective treatment for the prevention of rebleeding. Therefore, patients with aSAH should undergo emergency aneurysm repair.

Rebleeding is associated with a higher rate of other complications and worse outcomes [14]. The mortality associated with rebleeding is reported to be as high as 70 percent.

One of the most debilitating complication of aSAH is DCI occuring in approximately 30 percent of patients with aSAH, typically between 4 and 14 days after symptom onset [15]. 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 [16]. DCI is currently understood as a multifactorial process that evolves over time. The first 24 to 48 hours after ictus are referred to as the early brain injury phase, largely characterized by the sequelae of increased intracranial pressure and transient global ischemia during ictus. Cerebral edema, blood-brain barrier (BBB) disruption, sympathetic nervous system activation, autoregulatory failure, microthrombosis, spreading depolarizations (SDs), and inflammation have all been observed during this period [17]. Over time, the extravasated blood begins to aggravate and modulate the same core factors, culminating in the clinical manifestation of DCI around 4 to 10 days post-SAH [18].

Inability of cerebral perfusion to match metabolic demand is the ultimate cause of DCI; thus any pathological event that decreases perfusion or increases metabolic demand can contribute to DCI [19].

In the last decade cerebral vasospasm (CV) was assumed to be most common trigger of DCI. The exact mechanism is not clarified yet, but CV was believed to be produced by spasmogenic substances generated during the lysis of subarachnoid blood. Likelihood of CV may depend on the severity of bleeding and its proximity to the major intracerebral blood vessels.

Recently some other mechanisms have been revealed other than CV, that may contribute to DCI. The longlasting influence of the CV-centered approach to DCI research has been shifted to the examination of vascular dysfunction, neuroinflammation, and spreading depolarization associated with aSAH.

Oral administration of the dihydropyridine-type calcium channel blocker nimodipine is the only treatment with consistent, high-quality evidence for decreasing DCI [20] and is now standard of care in patients with aSAH, although these results are principally driven by 1 large trial [21]. Importantly, those early studies showed oral nimodipine reduces DCI and improves outcomes without affecting CV[22], suggesting nimodipine may have important vessel-independent effects. A recent trial (NEWTON [Nimodipine Microparticles to Enhance Recovery While Reducing

Toxicity After Subarachnoid Hemorrhage]) of intraventricular nimodipine administration found no improvements over standard oral administration [23]. Generally, nimodipine is generally administered to all patient orally for at least 14 days. Transcranial doppler (TCD) ultrasound can be performed daily to examine velocity changes in the middle cerebral artery (MCA). TCD ultrasound is cheap and affective non-invasive way for controlling CV. If there is suspicion for clinically relevant CV, DSA can be performed to confirm the diagnosis.

The CONSCIOUS trials demonstrated that inhibition of the vasoconstrictive endothelin-1 pathway decreases vasospasm but has no effect functional outcomes [24]. These clinical trials clearly demonstrate that targeting vascular dysfunction through vasodilation alone is not sufficient to reduce DCI.

In order to prevent and successfully treat DCI the loss of autoregulation, microthrombosis, cortical spreading depression, and delayed cellular apoptosis have to be taken into account [25].

20 to 30 percent of patients with aSAH are affected by hydrocephalus. It usually presents within the first few minutes to hours after SAH [26]. It can also be a later complication. Hydrocephalus after aSAH is thought to be caused by obstruction of cerebrospinal fluid (CSF) flow by blood products, adhesions or by a reduction of CSF absorption at the arachnoid granulations[27]. Progressive deterioration in level of consciousness, ocular signs of elevated ICP (miosis, downward eye deviation, or restricted upgaze) may raise the concern of hydrocephalus. In this case, early head CT scan should be perform to detect ventricular dilatation. Management of elevated ICP include immediate CSF diversion with an external ventricular drain (EVD) or lumbar drainage, osmotic therapy and diuresis, hemicraniectomy and long term management with ventriculoperitoneal shunt insertion.

Acute epileptic seizures occur in 6 to 18 percent of patients with aSAH [28]. Risk factors include thick subarachnoid clot, intracerebral hemorrhage, delayed infarction, and aneurysm in the MCA. Seizures that occur prior to aneurysm treatment are often a sign of early rebleeding[29].

2.5. Prognosis and classification of aSAH

The aSAH is associated with a high overall mortality and morbidity rate. Approximately 18 percent of patients with aSAH die suddenly prior to even being evaluated in a hospital [30]. Among patients who reach the hospital alive, much of the subsequent early mortality is caused by the common complications of aSAH related to initial bleeding, rebleeding, CV and DCI, hydrocephalus, increased ICP, seizures, and cardiac complications [31]. The overall mortality of aSAH (45%) is unacceptably high in the modern era of medicine. Furthermore, 30% of

survivors have mild or severe neurological disability after the attack. Successful intervention do not guarantee satisfying recovery, 66% of patients will be not able to live as previously.

Several scales have been established in the context of aSAH to estimate severity and prognosis. Few of the widespread disease severity assessment scale in aSAH patients are the Hunt and Hess scale, World Federation of Neurosurgeons scale (WFNS) and modified Fisher score system (mFisher). The Hunt and Hess scale was intended as an index of surgical risk. The initial clinical grade correlates with the severity of hemorrhage. Although the Hunt and Hess scale is easy to administer, the classifications are arbitrary, and some patients may not be placed within a single grade. Unlike the Hunt and Hess scale, the WFNS scale uses objective terminology to assign grades [32]. However, it may be more complex to administer than the Hunt and Hess scale because it requires assessment of both motor function and GCS. WFNS scale 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. The localization, bleeding volume, ventricular involvment is taken into account. Hunt and Hess scale assess the clinical signs associated with aSAH.

A

Grade	Characteristics	Mortality Rate (%)
0	Unruptured aneurysm without symptoms	0
1	Asymptomatic or minimal headache and slight nuchal rigidity	1
1a	No acute meningeal or brain reaction but with fixed neurologic deficit	1
2	Moderate to severe headache, nuchal rigidity, no neurologic deficit other than cranial nerve palsy	5
3	Drowsy, confused, or mild focal deficit	19
4	Stupor, moderate to severe hemiparesis, possible early decerebrate rigidity, and vegetative disturbances	42
5	Deep coma, decerebrate rigidity, moribund	77

B

WFNS Grade	GCS Score	Motor Deficit
I	15	absent
II	14–13	absent
III	14–13	present
IV	12–7	present or absent
V	6–3	present or absent

*WFNS = World Federation of Neurological surgeons; SAH = subarachnoid hemorrhage; GCS = Glasgow Coma Scale.

C

Group	Hemorrhage on CT
1	No hemorrhage evident
2	Diffuse or vertical layer of subarachoid hemorrhage < 1 mm thick
3	Localized clot and/or vertical layer of subarachnoid hemorrhage ≥ 1 mm thick
4	Intracerebral or intreaventricular clot, with or without a diffuse subarachnoid hemorrhage

D

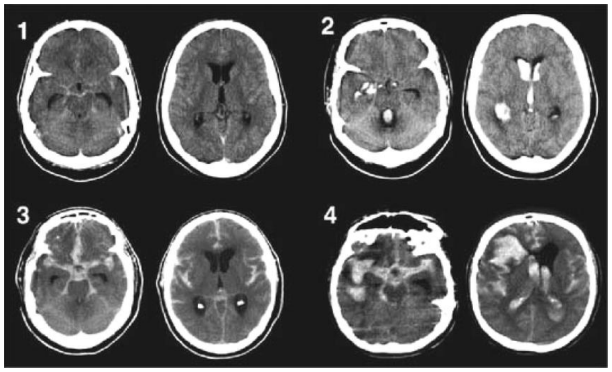

Table 2.2. *(A) Hunt and Hess scale (B), World Federation of Neurosurgeons scale (WFNS), (C) mFisher scale, (D) Subarachnoid Hemorrhage on native CT scan graded by mFisher scale*

Until these days no reliable prehospital prognostic factor has been discovered, that would help physicians determine patients with unfavorable outcome. Discovery of such prognostic factors would have substantial role in selecting the appropriate treatment regimen for each individual patient. Since patients with aSAH cause a substantial burden on the healthcare system, improvements in aSAH treatment remains a priority in the research field. We would like to contribute to these goals with our research.

3. BIOMARKERS IN aSAH

3.1. Definition

The National Institute of Health Biomarker Definitions Working Group (1988) defines a biological marker (biomarker) as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention'[33]. Sensitivity refers to the ability of a biomarker to detect the presence of a disease when the disease is present and specificity refers to its ability to exclude the disease when it is not present [34]. However, there is a use of limitation of recently explored blood biomarkers in the care of patients with aSAH.

3.2. Development of biomarkers

The detection of a novel marker is always preceded by a long (basic) research period. As an initial step in a biomarker study researchers should determine of a specific hypothesis for biomarker identification. This is the first phase, also called the discovery phase of biomarker development. The second stage is the qualification phase, when the choosen biomarker level is determined in the body fluid used as a diagnostic sample and it is demonstrated that different concentrations can be measured in the samples of individual patients and their controls. In the third verification phase, population-level studies are performed on healthy controls and the goal is to verify the specificity of the test. Finally, the validation phase provides opportunity for development and testing of a clinical assay, in which samples are taken from patients and their controls. In addition to the clinical sensitivity/specificity data, potential clinical applications of the new marker are also investigated [35,36].

Strategies for the development of aSAH biomarkers have taken several different approaches. One approach is to identify target molecules known to be involved in specific pathways in the process of cerebral hemorrhage, such as apoptosis, inflammation, hemorrheological abnormalities, cell death or oxidative damage. Other approach is the proteomic technology, also known as '-omic' technology. Proteomics is a continually advancing technology that encompasses a wide variety of techniques applied to the detection and analysis of proteins, such as mass spectrometry, sample enrichment techniques, quantitative protein labelling techniques, etc. Recent proteomics have studied ranges from global profiling of whole plasma or brain tissue to more focused analyses of vascular components including platelets, leukocytes and erythrocytes [37].

3.3. Role of biomarkers in aSAH

The pathophysiology of aSAH has played an important role in guiding biomarker research. Studying vascular dysfunction, neuroinflammation, and spreading depolarization associated with aSAH are the main research topics these days. The expanding use of biomarkers in the field of cerebral damage has made a substantial impact in our understanding of the pathophysiology of aSAH and the treatment. Several categories of biomarkers have been studied in cerebrovascular diseases – physical markers, imaging markers, electrophysiological markers, histological markers, genetic markers, systemic (serum) markers and neuronal markers. The primary focus of molecular biomarkers of cerebrovascular diseases has been their application to diagnosis and prediction of functional outcome.

3.4. Type of biomarkers in SAH

The model for a diagnostic marker is the cardiac isoenzyme test, troponin I and troponin T, which can detect myocardial ischemia with high sensitivity and specificity.

As for biomarkers for prediction of functional outcome, several markers has been studied in the context of aSAH.

(1) Neuron and astrocyte specific markers, including S100β, neuron-specific enolase (NSE), glial fibrillary acid protein (GFAP), apolipoprotein E (apoE) have been used as a prognostic adjunct tool, monitoring outcome following aSAH. Furthermore,

(2) Molecular adhesion and extracellular matrix markers [Matrix metalloproteinase-9 (MMP-9), Intracellular adhesion molecule-1 (ICAM-1), selectin],

(3) Biomarkers of vascular components and the clotting cascade (Vascular endothelial growth factor (VEGF), Endothelin-1, von Willebrand factor (vWf)) and

(4) Inflammatory markers [C-reactive protein (CRP), TNF-α, IL-1] have been investigated[38].

However, no biomarker has been found yet, that would be able to predict the outcome in aSAH patients with high specificity and sensitivity.

3.5. aSAH related neuroinflammatory biomarkers

Factors contributing to brain injury in aSAH are diverse. Following aneurysm rupture, injury can be divided into early and delayed stages, which include both systemic and cerebral factors. Inflammatory mechanisms play a key role in both phases [39]. The onset of aSAH elicits the activation of the thrombo-inflammatory cascade, causing ongoing neuroinflammation that is suspected to contribute to secondary complications, such as cerebral vasospasm and DCI [40]. A number of thrombo-inflammatory markers have been investigated in the context of cerebrovascular pathology, including cardiac troponin, S100B, high-sensitivity C-reactive protein (CRP), soluble CD40 ligand, tissue plasminogen activator (tPA), monocyte chemoattractant protein-1 (MCP-1), P-selectin and some members of the tumor necrosis superfamily (TNFSF) [41].

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 [42] LIGHT/TNFSF14 has been linked to a number of diseases such as multiple sclerosis, inflammatory bowel diseases, graft vs. host disease and atherosclerosis [43]. It can either costimulate or restrict the immune response binding to a subset of TNF superfamily receptors through diverse mechanisms [44]. 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 [45]. Several members of the TNF superfamily have been recently emerged in animal models of SAH [46]. 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 [47].

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 [48]. 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 [49,50.51]. In. contrast, at the level of the blood-brain-barrier (BBB) a pro-inflammatory readout of OSM have been observed [52]. To conclude, OSM can exhibit different functions depending on the variety of cell types that express the receptor and the cellular and molecular microenvironment [53]. 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 [54].

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 [55,56]. The serum level FABP3 were elevated within 3 hours after stroke [57] or myocardial ischemia [58], suggesting its role as a potential indicator of cellular injury. Zanier ER et al. showed [59] 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 [60]. 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 [61,62]. Ueland et al. showed [63] 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 [64,65], suggesting its key role in the pathogenesis of vascular diseases.

4. AIMS

The aim of the present thesis is to test if neuroinflammatory biomarkers released by pathyphysiological 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 Day 30 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.

5. 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;
- 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).

5.1. 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) with 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; or

(3) all previously mentioned criteria are met. The definition of DCI used in the study was based on an AHA recommendation published in 2010 [66]. For follow-up, we used structure telephone interviews performed by 1 doctor, blinded to clinical information.

5.2. 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.

5.3. 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 analyze 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 Day 30 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.

6. RESULTS

6.1. Patients characteristics

During the study period, 98 patients were initially assessed. 38 patients were excluded because of the reasons explained in **Figure 6.1.** 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; 7 (11.7%) 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.

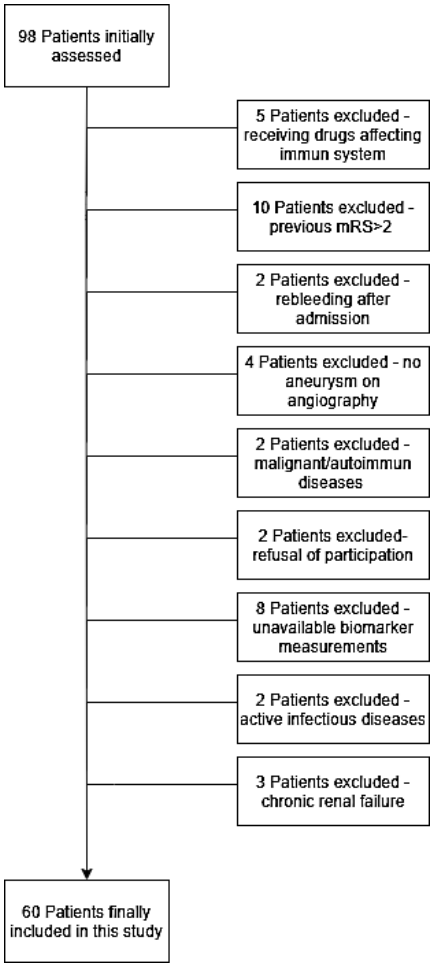

Figure 6.1.. *Flow chart illustrating excluded and included patients with aneurysmal subarachnoid hemorrhage*

6.2. 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).

6.3. 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 6.1.** 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 ventillation	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
Endocrin 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)	9.6 (7-12)	11.9 (8-19)	0.167

Table 6.1. *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.*

6.4. 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 unfavorable outcome, just for mortality alone.

Table 6.1. 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 6.2.)**.

	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 6.2. *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 6.3.**). 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 6.3. *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*

6.5. 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 6.4.)**. 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 6.2.).**

	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
Endocrin 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 6.4. *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).*

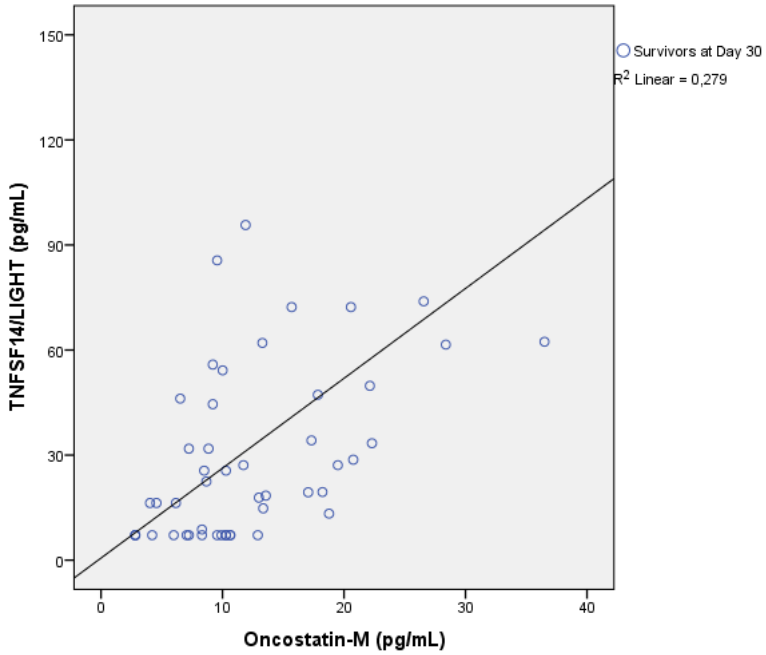

Figure 6.2. *Scatter-dot graph showing the positive correlation between serum LIGHT/TNFSF14 and Oncostatin-M levels measured 24 hours after the onset of symptoms in the survivor subgroup in patients with aneurysmal subarachnoid hemorrhage (p=0.001, 2-tailed).*

6.6. Independent predictor of mortality

The serum level of LIGHT/TNFSF14 within 24h 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 6.5.)**. The serum LIGHT/TNFSF14 with a cutoff value of >7.95 pg/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).

Day 30 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 6.5. *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)*

6.7. 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.

7. DISCUSSION

In the current study, we analyzed 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.

Our study demonstrated several novel findings:

(1) Serum FABP, LIGHT/ TNFSF14 and OSM levels were indeed elevated compared with healthy controls; while the serum CXCL-16 levels showed no differences between patients and controls

(2) There was an association between elevated serum level of both CXCL-16 and FABP3 and an unfavorable outcome at Day 30 after aSAH

(3) Serum CXCL-16 level with a cut-off level of > 446.7 pg/mL and serum FABP3 with a cut-off level of 3149.3 pg/mL was identified as an independent predictor of unfavorable outcome

(4) There was an independent association between LIGHT/TNFSF14 levels and survival of patients with aSAH in accordance with WFNS and modified Fisher scores;

(5) Serum level of LIGHT/TNFSF14 with a cutoff value of >7.95pg/ml was found to be an independent predictor of Day 30 survival in aSAH patients

(6) Significant correlation was observed between LIGHT/TNFSF14 and OSM in the sera of surviving patients.

(7) We also found that lower serum level of LIGHT/TNFSF14 was significantly associated with the incidence of DCI.

Several mechanisms are responsible for aSAH-related mortality. A recent review found age, loss of consciousness at ictus, admission Glasgow Coma Scale score, large aneurysm size, Acute Physiology and Chronic Health Evaluation II physiologic score, and admission Modified Fisher Scale score as predictors of mortality [67]. Besides, the systemic response to subarachnoid hemorrhage affecting the respiratory system (acute respiratory distress syndrome, ARDS, pulmonary edema), the heart (arrhythmias, contractility abnormalities), the fluid-electrolyte homeostasis, and the systemic inflammatory response syndrome can also influence the overall survival [68].

SAH cause brain injury via glutamate excitotoxicity, leading to an excessive Ca^{2+} influx and can induce an apoptotic cascade [69]. Wang et al. found that CSF levels of glutamate are significantly higher in patients with severe SAH (WFNS grade 4-5 and mFisher score 3-4) than those with less severe SAH (WFNS grade 1-3 and mFisher score 1-2)[57]. CXCL-16 can promote physiological neuroprotective mechanisms that counteract neuronal cell death due to ischemic and excitotoxic insults mediated by glutamate [70]. CXCL-16 acts directly on astrocytes to release soluble factors essential to mediate neuroprotection against excitotoxic damage due to excessive glutamate exposure [71]. 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 [72]. 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 [73] and in acute coronary syndrome [74]. 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 [75]; (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 [76]. Both apoptosis [77] [78] and damaged cerebral microvasculature [79] 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 [80]. 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 [81]. 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, Yilman et al. found that serum level of FABP3 showed significant association with Hunt Hess score and Fisher score in a small cohort of patient with SAH [79]. In a study by Zanier et al., the CSF level of FABP3 showed significantly higher peak levels in patients with severe SAH than mild SAH patients [59]. A study by Wunderlich *et al.* 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 [57]. Importantly,

FABP3 is a more sensitive marker for minor brain injury than the commonly used markers such as S100B or NSE [55]. Moreover, it has an excellent ability to differentiate between CT-positive and CT-negative mild TBI patients [82]. FABP3 is also known to be a sensitive marker in acute coronary events and cardiac failure [83,84,85]. 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 [86]. Its serum concentration remains increased for days after ischemic stroke [57]; 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) [87]. FABP3 can bind the 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 [75].

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 [57,82]. 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. Scholz et al [88] found enhanced plasma levels of LIGHT/TNFSF14 in unstable angina. Chien-Yi Hsu et al [89] demonstrated that increased 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 [90]. 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 [91]. 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 [92]. LIGHT/TNFSF14 engages two cellular signaling receptors, lymphotoxin receptor (LTR) and HVEM, and is inactivated by Decoy receptor-3 (DcR3) [93]. Circulating LIGHT/ TNFSF14 is unbound to DcR3. This "free LIGHT/TNFSF14" is the soluble form that retains receptor-binding activity [43]. 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 [79]. Another member of the TNF superfamily, TWEAK, has significantly higher serum levels in patient with poor outcome after SAH [15]. Like LIGHT, two forms of TWEAK are known as the soluble form and the membrane-bound form [94]. 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 [95]. Level of several types of MMPs was significantly higher in aSAH cases compared with controls [96] and subarachnoid bleeding significantly upregulates both the expression and activity of matrix metalloproteinase-9 (MMP-9) in blood and CSF [97,98,99]. Several experimental models suggest that MMP-9 activity mediates blood-brain barrier breakdown and subsequent vasogenic edema following SAH [100]. 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 [101]. Its neutralizing effect on LIGHT/TNFSF14 [102] may contribute to the detrimental outcome after aSAH. Several studies revealed increased serum DcR3 levels in some chronic inflammatory conditions, such as sepsis, [103] acute respiratory distress syndrome, [104] and cardiovascular disease [105]. Interleukin-6 upregulates DcR3 expression [106]. On the other hand, elevated serum concentration of IL-6 was detected in patients with unfavorable outcome of SAH [107]. 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. Krause et al [108] provided evidence 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 [109].

Importantly, OSM concentration showed a strong positive correlation with the level of LIGHT/TNFSF14 in the sera of surviving patients. Guo et al proposed that OSM may induce JAK2/STAT3-mediated neuroprotection during ischemic stroke [50]. The neuroprotective effect induced by STAT3 activation has been reported by many authors [110]. Weiss et al [111] found 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. Han et al 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 [112]. Mikami et al [113] 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 [114]. 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 [25]. 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 [115]. Microthrombi also contribute to the development of DCI. The absence of LIGHT/ TNFSF14 from the cell surface of platelets causes rapid platelet aggregation [116]. 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 [117].

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.

8. 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 Day 30 favorable clinical outcome. Morover, a positive correlation emerged between CXCL-16 and FABP3 levels in our cohort.

Ad Aim 3.

The elevation of the serum level 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.

9. LIMITATIONS

First, our study sample was relatively small and the duration of follow-up was short. Results need to be confirmed on a larger sample size and longer follow up of confirmed aSAH. Secondly, sampling at multiple time points instead of a single shot could clarify whether the kinetics of CXCL-16, FABP3, LIGHT/TNFSF14 and OSM differ in various subgroups of aSAH patients. Thirdly, the rigid inclusion/exclusion criteria limit the generalizability of the study, while a detailed assessment of the functional outcome in the case of SAH could have provided more information than using the mRS scale alone. Beneficial would be to measure the concentration of the free form found in serum and the memban-bound form simultaneously, and draw conclusions from their relationship. Other important limitations of the article: short follow-up period, lack of data on soluble vs. membrane-bound forms of the investigated biomarkers, and absence of patients that received surgical clipping which may also limit generalizability of our cohort. Additionally, it would be beneficial to investigate the role of other inflammatory biomarkers that are involved in the same signal pathways or other members of the molecule superfamilies in the context of SAH.

10. FURTHER AIMS AND PERSPECTIVES

A paradigm shift is currently going on from testing single picked markers towards high throughput genomics or proteomics identifying biomarker signatures in this field. These would more likely result in a comprehensive identification and mapping of entire biological pathways causally involved in SAH pathogenesis, and complications such as DCI. Based on these findings, a personalized therapeutic intervention can ideally be guided leading to a favorable outcome in patients with aSAH. Beside conventional CNS specific markers (e.g. S100B, GFAP etc), novel molecules should be involved to create clusters of patients with different risk for complications and adverse events. Indeed, a deeper insight of the pathophysiological roles of such markers will be needed in the future. In our view, a multiple-marker based approach will emerge as a future perspective to provide a personalized care for patients with aSAH. By the analogy of precision oncology, disease and risk specific markers should be individually determined and processed by arteficial intelligence (AI) in networks supporting clinical decisions that may result in a favorable clinical outcome.

Our further aim is to investigate several other inflammatory biomarker in the acute and subacute phase of SAH and measure the initial, on day3/6/9/12 serum level of these molecules, and follow their serum levels alterations. We are going to make a large data pool, and summarizes the predictive value of the biomarker levels regarding the outcome of SAH and development of DCI. We are eager to establish a prehospital point of care testing system for measuring serum levels of these predictive molecules in order to individualize the treatment. With the assistance of Artificial Intelligence (AI) based on the biomarker levels we may be able to diagnose SAH patients prehospitally, and choose those how will benefit the most from neurointerventional procedures.

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12. LIST OF PUBLICATION

12.1. 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**

12.2. 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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ORIGINAL ARTICLE

Increased level of LIGHT/TNFSF14 is associated with survival in aneurysmal subarachnoid hemorrhage

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Abstract

Objectives: Multiple cytokines have been implicated in aneurysmal subarachnoid hemorrhage (aSAH), but tumor necrosis factor superfamily 14 (LIGHT/TNFSF14) and oncostatin-M (OSM) have not been previously explored.

Aims of the Study: The primary objective of this study was to examine the relationship between TNFSF14 and OSM levels and survival. Our secondary goal was to investigate a potential association between these markers and the incidence of delayed cerebral ischemia (DCI).

Materials & Methods: We consecutively recruited 60 patients with a clinical diagnosis of aSAH. LIGHT/TNFSF14 and OSM serum concentrations were determined by ELISA. The primary endpoint was survival at Day 30, while development of DCI was assessed as secondary outcome.

Results: Patients had significantly higher levels of both markers than the control group (median of LIGHT: 18.1 pg/ml vs. 7 pg/ml; $p = 0.01$; median of OSM: 10.3 pg/ml vs. 2.8 pg/ml, $p < 0.001$). Significantly lower serum level of LIGHT/TNFSF14 was found in nonsurviving patients ($n = 9$) compared with survivors ($n = 51$; $p = 0.011$). Based on ROC analysis, serum LIGHT/TNFSF14 with a cutoff value of >7.95 pg/ml predicted 30-day survival with a sensitivity of 71% and specificity of 78% (Area: 0.763; 95% CI: 0.604–0.921, $p = 0.013$). In addition, it was also a predictor of DCI with a sensitivity of 72.7% and a specificity of 62.5% (AUC: 0.702; 95% CI: 0.555–0.849, $p = 0.018$). Based on binary logistic regression analysis, LIGHT/TNFSF14 was found to be independently associated with 30-day mortality, but not with DCI.

Conclusion: In this cohort, a higher serum level of LIGHT/TNFSF14 was associated with increased survival of patients with aSAH.

KEYWORDS
LIGHT, mortality, subarachnoid hemorrhage, TNFSF14

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1 | INTRODUCTION

Aneurysmal subarachnoid hemorrhage (aSAH) is associated with unacceptably high mortality.[1] Factors contributing to brain injury in SAH are diverse. Following aneurysm rupture, injury can be divided into early and delayed stages, which include both systemic and cerebral factors. Inflammatory mechanisms play a key role in both phases.[2] The onset of aSAH elicits the activation of the thrombo-inflammatory cascade, causing ongoing neuroinflammation that is suspected to contribute to secondary complications, such as vasospasm and delayed cerebral ischemia.[3] 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.[4] LIGHT/TNFSF14 has been linked to a number of diseases such as multiple sclerosis, inflammatory bowel diseases, graft vs. host disease and atherosclerosis.[5] It can either costimulate or restrict the immune response through diverse mechanisms.[6] A number of thrombo-inflammatory markers have been investigated in the context of cerebrovascular pathology.[7] Several TNF superfamily molecules play an important role in immunity and activation, proliferation, differentiation, or even migration of immune cells into the central nervous system.[8] Several members of the TNF superfamily have been recently emerged in animal models of subarachnoid hemorrhage.[9] In addition, TNFSF12 was found to be a potential biomarker for outcome prediction in patients with subarachnoid hemorrhage.[10]

Oncostatin-M (OSM) is a member of the glycoprotein 130 (or IL-6/LIFR—leukemia inhibitory factor receptor) cytokine family. Several studies reported that it has a beneficial effect in many central nervous system diseases such as demyelinating diseases, ischemic stroke, and spinal cord injury.[11–13] 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.[14] Based on these research data, we decided to examine the systemic concentration of LIGHT and OSM in patients suffering from aSAH compared with controls, and particularly their influence on the outcome. Besides, we aimed to explore the association between the serum concentration of such molecules and delayed cerebral ischemia (DCI).

2 | METHODS

In this prospective, observatory study, consecutive aSAH patients were enrolled at the Department of Neurosurgery, University of Pecs, between October 2018 and September 2020. Inclusion criteria for the study were (1) age >18 years; (2) clinical history of aneurysmal SAH with an aneurysm identified on CT angiography or digital subtraction angiography within 24 h after symptom onset; and (3) informed consent obtained from the patient or legal representative. Exclusion criteria were (1) rebleeding with a minimum 3-point drop in GCS or a 4-point increase in NIHSS scale after admission and before endovascular treatment; (2) malignant or autoimmune disorder; (3) 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; (4) estimated glomerular filtration rate, eGFR <50 and/or creatinine >120 μmol/L at two distinct measurements; (5) evidence of concomitant coronary syndrome (if all of the followings are met: troponin-I value >14 ng/L; typical clinical symptoms; characteristic ECG changes); (6) unavailable biomarker measurements; (7) refusal of participation; and (8) 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 SAH patients was assessed on hospital admission using the World Federation of Neurosurgeons scale (WFNS) and modified Fischer score system. 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).

2.1 | Outcome

We aimed to evaluate the association of biomarkers with (1) mortality at Day 30 as primary endpoint and (2) delayed cerebral ischemia (DCI) as secondary endpoint. 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) 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; or (3) all previously mentioned criteria are met. The definition of DCI used in the study was based on an AHA recommendation published in 2010.[15] Functional outcome at day 30 was also assessed based on mRS.

2.2 | 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 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 and 1.6 pg/ml for LIGHT/TNFSF14 and 0.6 pg/ml for OSM.

2.3 | 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 analyze 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 (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.

3 | RESULTS

Initially, a total number of 98 patients with aSAH were assessed, but 38 patients were excluded because of the reasons listed in Figure 1. This study finally included 60 aSAH patients, and 21 healthy subjects were enrolled as controls. Neither the gender (female: SAH, 53,3% vs. control 61.9%, p = 0.496), nor the age (58.1 ± 11.2 vs. 57 ± 17, p = 0.686) showed significant difference between patients and healthy controls. Both 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 LIGHT: 18.1, IQR: 7.1–46.7 vs. 7.1, 7.1–7.3 and median of OSM: 10.3, 7.5–16.4 vs. 2.8, 2.8–4.9, p < 0.001, respectively).

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, the OSM level showed no significant difference.

Comparison of demographic and clinical parameters between nonsurviving (n = 9) and surviving (n = 51) patients are shown in Table 1. 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). When a statistical analysis was performed with favorable outcome defined as mRS 0–2, no significant difference in serum TNFSF14 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 (favorable, median 24 pg/ml (7–59) vs. unfavorable 10.2 pg/ml (7.1–28), p = 0.048). Moreover, a significant correlation was observed between LIGHT/TNFSF14 and OSM in the sera of surviving patients (p < 0.001) (Figure 2).

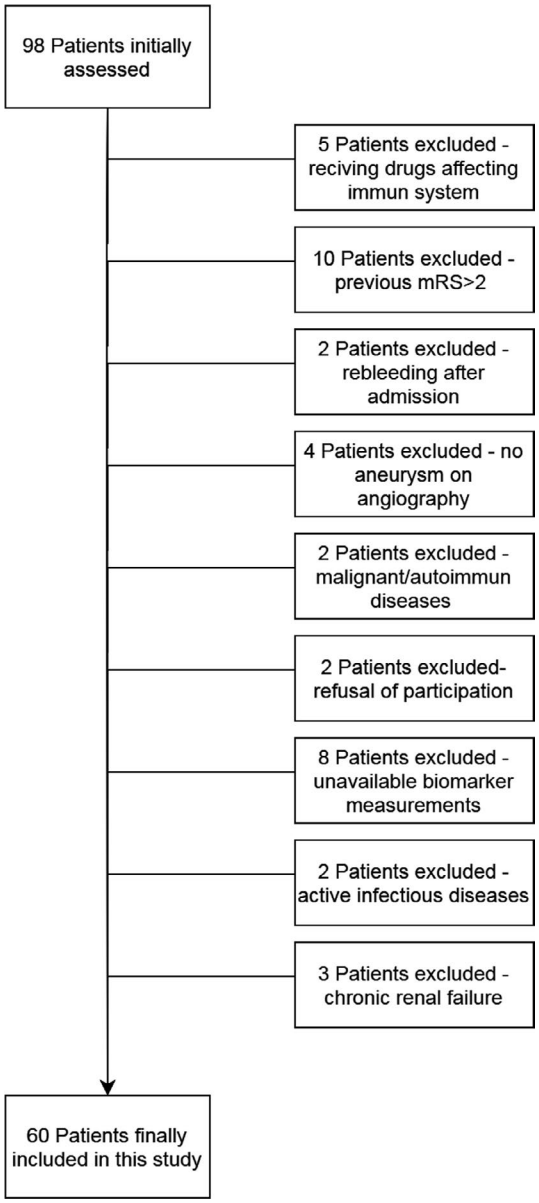

FIGURE 1 Flowchart illustrating excluded and included patients with aneurysmal subarachnoid hemorrhage

3.1 | Independent predictor of mortality

Based on ROC analysis, serum LIGHT/TNFSF14 with a cutoff value of >7.95 pg/ml predicted 30-day 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

TABLE 1 Comparison of clinical and biochemical characteristics between nonsurvivors and survivors with aneurysmal subarachnoid hemorrhage

	Nonsurvivors (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*
mFischer score on admission	4 (4)	3 (2–4)	0.005*
Aneurysmal location			
Posterior communication artery	2 (22.2%)	8 (15.7%)	
Internal carotid artery	2 (22.2%)	8 (15.7%)	
Anterior communication artery	2 (22.2%)	15 (29.4%)	
Middle cerebral artery	1 (11.1%)	8 (15.7%)	
Anterior cerebral artery	1 (11.1%)	6 (11.8%)	
Posterior circulation	1 (11.1%)	6 (11.8%)	
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

Note: The categorical variables are presented as frequency and percentage, and the continuous variables are presented as mean ±standard deviation or median (percentile 25–75).

Abbreviations: mFischer, modified Fischer; CRP, C-reactive protein; NLR, neutrophil-lymphocyte ratio; PLR, platelet-lymphocyte ratio; LIGHT/TNFSF14, Tumor necrosis factor superfamily 14; WFNS indicates World Federation of Neurological Surgeons.

* Indicates significant correlation ($p < 0.05$).

as mFischer score or WFNS score, the two-variable ROC analysis explored improved predictive power of mortality of both combination (LIGHT+mFischer, Area: 0.878, 95% CI: 0.78–0.98; $p < 0.001$, and LIGHT+WFNS, Area: 0.829, 95% CI: 0.72–0.94; $p = 0.002$). However,

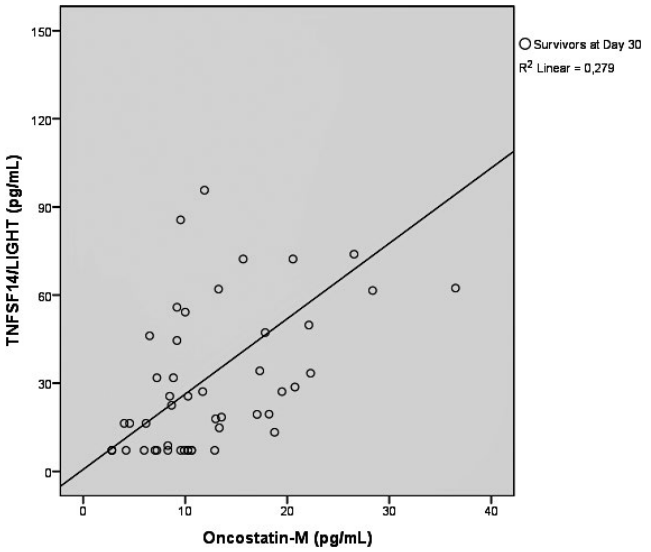

FIGURE 2 Scatter-dot graph showing the positive correlation between serum LIGHT/TNFSF14 and Oncostatin-M levels measured 24 h after the onset of symptoms in the survivor subgroup in patients with aneurysmal subarachnoid hemorrhage ($p = 0.001$, 2-tailed)

the combination of LIGHT+presence of DCI and LIGHT+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).

3.2 | Prediction of 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 7.95 pg/ml (AUC of 0.702, 95% CI: 0.555–0.849; $p = 0.018$) with a 72.7% sensitivity and 62.5% specificity.

The serum level of LIGHT/TNFSF14 within 24 h and mFischer score assessed on admission were independently associated with 30-day survival, whereas age, gender, DCI, and in-hospital infection rate were not (Table 2).

4 | DISCUSSION

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

Our study demonstrated several novel findings: (i) serum LIGHT/TNFSF14 and OSM levels were indeed elevated compared with

TABLE 2 Binary logistic regression analysis for variables independently associated with survival (mRS<6) on day 30 in patients with aneurysmal subarachnoid hemorrhage

	30 day survival			
Variables	B	Odds ratio	95% CI	*p*-value
LIGHT/TNFSF14	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
mFischer 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

Abbreviations: DCI, delayed cerebral ischemia; LIGHT/TNFSF14, Tumor necrosis factor superfamily 14; mFischer score, modified Fischer score.
* Indicates significant correlation ($p < 0.05$).

healthy controls; (ii) there was an independent association between LIGHT/TNFSF14 levels and survival of patients with aSAH in accordance with WFNS and modified Fisher scores; (iii) a higher serum level of LIGHT/TNFSF14 was found to be an independent predictor of 30-day survival in aSAH patients. We also found that lower serum level of LIGHT/TNFSF14 was significantly associated with the incidence of DCI.

Several mechanisms are responsible for SAH-related mortality. A recent review found age, loss of consciousness at ictus, admission Glasgow Coma Scale score, large aneurysm size, Acute Physiology and Chronic Health Evaluation II physiologic score, and admission Modified Fisher Scale score as predictors of mortality.[16] Besides, the systemic response to subarachnoid hemorrhage affecting the respiratory system (acute respiratory distress syndrome, ARDS, pulmonary edema), the heart (arrhythmias, contractility abnormalities), the fluid-electrolyte homeostasis, and the systemic inflammatory response syndrome can also influence the overall survival.[4]

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. Scholz et al[17] found enhanced plasma levels of LIGHT/TNFSF14 in unstable angina. Chien-Yi Hsu et al[18] demonstrated that increased 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.[19] 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.[20] 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.[21] LIGHT/TNFSF14 engages two cellular signaling receptors, lymphotoxin receptor (LTR) and HVEM, and is inactivated by Decoy receptor-3 (DcR3).[22] Circulating LIGHT/TNFSF14 is unbound to DcR3. This "free LIGHT/TNFSF14" is the soluble form that retains receptor-binding activity.[5] 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.[20] Another member of the TNF superfamily, TWEAK, has significantly higher serum levels in patient with poor outcome after SAH[15] Like LIGHT, two forms of TWEAK are known as the soluble form and the membrane-bound form.[23] 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.[24] Level of several types of MMPs was significantly higher in aSAH cases compared with controls[25] and subarachnoid bleeding significantly upregulates both the expression and activity of matrix metalloproteinase-9 (MMP-9) in blood and CSF.[26–28] Several experimental models suggest that MMP-9 activity mediates blood-brain barrier breakdown and subsequent vasogenic edema following SAH.[29] 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.[30] Its neutralizing effect on LIGHT/TNFSF14[31] may contribute to the detrimental outcome after aSAH. Several studies revealed increased serum DcR3 levels in some chronic inflammatory conditions, such as sepsis,[32] acute respiratory distress syndrome,[33] and cardiovascular disease.[34] Interleukin-6 upregulates DcR3 expression.[35] On the other hand, elevated serum concentration of IL-6 was detected in patients with unfavorable outcome of SAH.[36] 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. Krause et al[37] provided evidence 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. Yang et al[38] found that LIGHT/TNFSF14 played a protective role in cisplatin-induced acute kidney injury (Cis-AKI) in mice, which was considered to be related to downregulated inflammatory molecules and decreased apoptosis. In a mouse model of

colitis, LIGHT/TNFSF14 played an important role in protection from inflammation.[39] In addition, more severe disease pathogenesis was observed in LIGHT/TNFSF14-deficient mice.[39]

Importantly, OSM concentration showed a strong positive correlation with the level of LIGHT/TNFSF14 in the sera of surviving patients. Guo et al proposed that OSM may induce JAK2/STAT3-mediated neuroprotection during ischemic stroke.[8] The neuroprotective effect induced by STAT3 activation has been reported by many authors.[33,40] Weiss et al[41] found 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.[41] Han et al 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.[42] Mikami et al[43] 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 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. 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.[2] We previously described that LIGHT/TNFSF14 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.[44] Microthrombi also contribute to the development of DCI. The absence of LIGHT/TNFSF14 from the cell surface of platelets causes rapid platelet aggregation.[45] 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.[46] In our study, the level of soluble LIGHT/TNFSF14 reflects just an early state of aSAH as sampling was done within the first 24 h. The kinetics of LIGHT/TNFSF14 in the systemic circulation should be explored in the future.

Here, we observed an association between LIGHT/TNFSF14 level measured in the subacute phase and 30-day survival after aSAH. In addition, a positive correlation was also found between LIGHT/TNFSF14 and OSM levels in survivals. Presumably, the lack of elevation of LIGHT/TNFSF14 in nonsurvivors may suggest its potential protective effect independently from complications such as development of DCI. 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 LIGHT/TNFSF14 and OSM system should be explored in future SAH trials providing potential therapeutic targets.

In summary, the protective role of LIGHT/TNFSF14 emerged from this cohort. The pro- and anti-inflammatory properties of the LIGHT/TNFSF14 showing the Janus face of this molecule have been reported under different clinical conditions. Therefore, larger prospective studies involving patients with aSAH would justify clarification of its clinical utility.

5 | LIMITATIONS

First, our study sample was relatively small and the duration of follow-up was short. Results need to be confirmed on a larger sample size of confirmed aSAH. Secondly, sampling at multiple time points instead of a single shot could clarify whether the kinetics of LIGHT/TNFSF14 and OSM differ in various subgroups of aSAH patients. Thirdly, the rigid inclusion/exclusion criteria limit the generalizability of the study, while a detailed assessment of the functional outcome in the case of SAH could have provided more information than the mortality alone. Other important limitations of the article: short follow-up period, lack of data on soluble vs. membrane-bound LIGHT/TNFSF14, and absence of patients that received surgical clipping which may also limit generalizability.

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CONFLICTS OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Fatty Acid-Binding Protein 3 and CXC-Chemokine Ligand 16 are Associated with Unfavorable Outcome in Aneurysmal Subarachnoid Hemorrhage

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Background: Aneurysmal subarachnoid hemorrhage (aSAH) is associated with activation of the inflammatory cascade contributing to unfavorable outcome and secondary complications, such as delayed cerebral ischemia (DCI). Both fatty acid−binding protein 3 (FABP3) and CXC-chemokine ligand 16 (CXCL-16) have been linked to vascular inflammation and cellular death. The authors aimed to assess the 30-day prognostic value of serum levels of FABP3 and CXCL-16 and explore their associations with DCI in aSAH patients. *Methods:* A total of 60 patients with aSAH were prospectively enrolled. Sampling for markers was done at 24 hours after the index event. FABP3 and CXCL-16 serum concentrations were determined by MilliPlex multiplex immunoassay method. The primary endpoint was unfavorable outcome at Day 30 based on the modified Rankin Scale. *Results:* Both FABP3 and CXCL-16 levels were significantly elevated in patients with unfavorable outcome compared to those with favorable outcome after aSAH (FABP3: 2133 pg/mL, IQR: 1053-4567 vs. 3773, 3295-13116; $p<0.003$ and CXCL-16: 384 pg/mL, 313-502 vs. 498, 456-62, $p<0.001$). 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 binary logistic regression analysis, serum CXCL-16 with a cut-off level >446.7 ng/L independently predicted Day 30 unfavorable outcome with a sensitivity of 81% and a specificity of 62%. Neither CXCL-16 nor FABP3 showed a significant correlation with DCI. *Conclusion:* Early FABP3 and CXCL-16 levels are significantly associated with poor 30-day outcome in patients with aSAH.

Key Words: Subarachnoid hemorrhage—FABP3—CXCL-16—Unfavorable outcome

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Abbreviations: aSAH, aneurysmal subarachnoid hemorrhage; FABP3, fatty acid-binding protein 3; CXCL-16, CXC-chemokine ligand 16; DCI, delayed cerebral ischemia; AUC, area under the curve; IQR, interquartile range; IFN, interferon; DSA, digital subtraction angiography; GCS, Glasgow coma scale; NIHSS, National Institute of Health Stroke Scale; mRS, modified Rankin score; eGFR, estimated glomerular filtration rate; ECG, electrocardiogram; CTA, computer tomography angiography; WFNS, World Federetaion of Neurological Societies; DWI, diffusion weighted image; NLR, neutrophil - lymphocyte ratio; CRP, C-reactive protein; ROC, Receiver operating characteristic; CNS, central nervous system; CSF, cerebrospinal fluid; TBI, traumatic brain injury; NT-proBNP, N-terminal pro brain natriuretic peptide; NSE, neuron specific enolase

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Introduction

Aneurysmal subarachnoidal hemorrhage (aSAH) is associated with unacceptably high mortality.[1] Factors contributing to brain injury in SAH are diverse. Following aneurysm rupture, injury can be divided into early and delayed stages, including systemic and cerebral factors. Inflammatory mechanisms play a vital role in both phases.[2] The onset of aSAH elicits activation of the inflammatory cascade, and ongoing neuroinflammation is suspected of contributing to secondary complications, such as vasospasm and delayed cerebral ischemia (DCI).[3]

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.[4-5] The serum level of FABP3 was elevated within 3 hours after stroke[6] or myocardial ischemia[7], suggesting its role as a potential indicator of cellular injury. Zanier ER et al. showed[8] that aSAH patients with clinical sign of vasospasm or unfavourable neurological outcome at 30 days have higher FABP3 levels.

Chemokines are known to play an important role in atherogenesis and vascular inflammation.[9] The novel CXC-chemokine ligand 16 (CXCL-16) is both an interferon (IFN) γ regulated chemokine and a scavenger receptor that is up-regulated in macrophages.[10-11] Ueland et al. showed[12] that the increase in plasma levels of CXCL-16 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[13-14], suggesting its key role in the pathogenesis of vascular diseases.

The aim of our study was to assess the 30-day prognostic value of serum levels of FABP3 and CXCL-16 measured within 24 hours in patients with aneurysmal subarachnoidal hemorrhage. We also analyzed the link between the serum concentration of such molecules and delayed cerebral ischemia (DCI).

Methods

This prospective observational study was carried out based on a formerly recruited adatabase involving consecutive aSAH patients, who were admitted at the Department of Neurosurgery, University of Pecs between October 2018 and September 2020 (15). Inclusion criteria for the study were: (1) age > 18 years; (2) clinical history of aneurysmal SAH with an aneurysm noted on CT angiography (CTA) or digital subtraction angiography (DSA) within 24 hours after symptom onset; (3) informed consent obtained from each patient or legal representative. Exclusion criteria were: (1) rebleeding with a minimum 3-point drop in Glasgow Coma Scale (GCS) or a 4-point increase in the National Institute of Health Stroke Scale (NIHSS) after admission and before endovascular treatment; (2) malignant or autoimmune disorder; (3) active infectious diseases (symptoms of infection with fever, elevated C-reactive protein and or procalcitonin, and a positive diagnostic test such as chest X-ray or urine test; (4) estimated glomerular filtration rate (eGFR) <50 and/or creatinine >120 μmol/l at two distinct measurements; (5) evidence for concomitant coronary syndrome (if all of the followings are met: troponin-I value > 14 ng/L; adequate clinical symptoms; characteristic ECG changes); (6) unavailable biomarker measurements; (7) refusal of participation; (8) 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 SAH patients was assessed on hospital admission using the World Federation of Neurosurgeons scale (WFNS) and modified Fischer score system. Demography, clinical and laboratory data, as well as medical history, were also recorded. Other relevant variables such as the need for mechanical ventilation, decompressive craniotomy or ventricular drainage were also explored.

The Local Ethics Committee approved the study protocol at the 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).

Outcome

We aimed to evaluate the association of biomarkers with: (i) unfavorable outcome (mRS score 3-6) at Day 30 as the primary endpoint; and (ii) delayed cerebral ischemia (DCI) as the secondary endpoint. Patients were classified as complicated with DCI if (1) presenting with a change in the level of consciousness (a decrease of at least 2 points in the GCS) or an increase of more than 2 points in NIHSS score or development of new focal deficit lasting for at least 1 hour and not explained by other factors; (2) having a new DWI lesion on MRI obtained after DCI suspected; (3) or if all previously mentioned criteria were met.

Sample collection

Arterial blood samples were drawn from each patient on admission within 24 hours after symptom onset, immediately before neurointervention. The samples were immediately centrifuged at 400 r/min for 15 minutes. The supernatant was stored at −80°C until analysis. FABP3 and CXCL-16 concentrations were determined using MILLIPLEX MAP Human Cardiovascular Disease Magnetic Bead Panel 1 - Cardiovascular Disease Multiplex Assay (Merck KGaA, Darmstadt, Germany). The same assay also measured Troponin-I to exclude ongoing

coronary syndrome. All samples were processed by the same technicians using the same equipment and blinded to all clinical data. The detection limit for the assay was 43 pg/mL for troponin-I, 23.7 pg/mL for FABP3 and 11.9 pg/mL for CXCL-16.

Statistical analysis

Data were evaluated using SPSS (version 11.5; IBM, Armonk, NY, USA). The Kolmogorov-Smirnov test was applied to check for normality. The chi-square test for categorical data and Student t-test for continuous data were used to analyze demographic and clinical factors. Non-normally distributed data were presented as median and interquartile range and compared with the Mann-Whitney test. The cut-off values with the best sensitivity and specificity of FABP3 and CXCL-16 (n=60) to determine 30-day survival and delayed cerebral ischemia were calculated by the receiver operator curve (ROC) analysis. Correlation analysis was performed calculating Spearman's correlation coefficient (rho). To explore the independent predictors of 30-days poor functional outcome, a binary logistic regression was used. A p-value < 0.05 was considered statistically significant.

Results

Patients characteristics

During the study period, 98 patients were initially assessed. 38 patients were excluded because of the reasons explained in Fig. 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; 7 (11.7%) required mechanical ventilation during hospitalization and DCI developed in 16 (26.7%) cases. The mean serum C-reactive protein (CRP) level was 49.5±63 mg/L; the mean neutrophil-lymphocyte ratio (NLR) was 7.7±5, and the mean serum creatinine level was 63.5±21.6 μmol/L.

Biomarker levels

The serum FABP3 levels measured at 24 hours after symptom onset were significantly elevated in patients

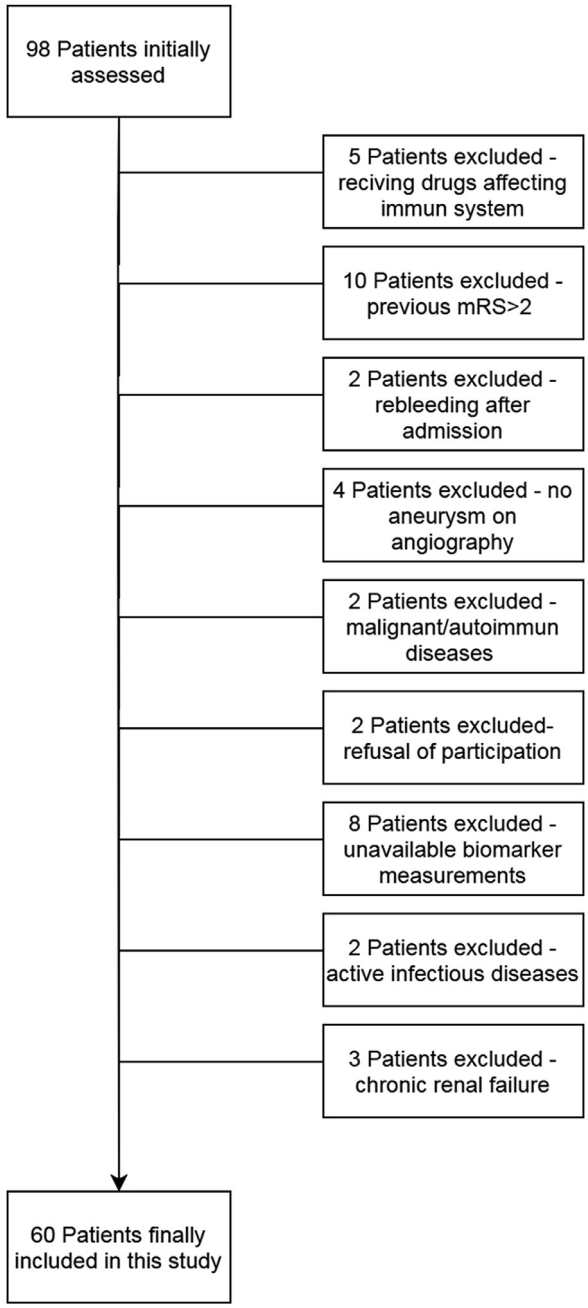

Fig. 1. *Flowchart illustrating excluded and included patients with aneurysmal subarachnoid hemorrhage.*

(3325 pg/mL, IQR: 1548-5297) compared to healthy controls (1373 pg/mL, 811-2498, p=0.003). The serum CXCL-16 levels showed no differences between patients and controls (462 pg/mL, 360-563 vs. 431 pg/mL, 307-498, p=0.242). The results of sub-population analysis for both markers showed that the patients with an unfavorable outcome revealed higher FABP3 and CXCL-16 concentrations (p=0.003, p<0.001, respectively) (Table 1). CXCL-16 and FABP3 were significantly correlated with a number of factors in univariate analysis (as shown in Table 2). Variables associated with FABP3 included mFisher score,

Table 1. *Comparison of clinical and biochemical characteristics between patients with favaorable (mRS score 0-2) and unfavourable (mRS 3-6) outcome at discharge with aneurysmal subarachnoid hemorrhage.*

	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	3 (9.1%)	6 (23.1%)	0.138
WFNS grade on admission	2 (1-2)	4 (3-5)	<0.001*
mFischer score on admission	3 (2-3)	4 (3-4)	0.002*
Aneurysmal location			
Posterior communication artery	2 (22.2%)	8 (15.7%)	
Internal carotid artery	2 (22.2%)	8 (15.7%)	
Anterior communication artery	2 (22.2%)	15 (29.4%)	
Middle cerebral artery	1 (11.1%)	8 (15.7%)	
Anterior cerebral artery	1 (11.1%)	6 (11.8%)	
Posterior circulation	1 (11.1%)	6 (11.8%)	
Ventricular drainage	7 (20.6%)	17 (65.4%)	<0.001*
Mechanical ventillation	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 ischaemia	5 (14.7%)	11 (42.3%)	0.017
Endocrin disorder during hospitalization	8 (24.2%)	5 (19.2%)	0.645
CXCL-16 (pg/mL)	384 (313-502)	498 (456-623)	<0.001*
FABP3 (pg/mL)	2133 (1053-4567)	3773 (3295-13116)	0.003*

Table 1. 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; mFischer, modified Fischer; 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; significance is indicated by asterisks (*)

WFNS score, serum creatinine, serum CRP, platelet count and NLR both correlated with mechanical ventilation, ventricular drainage and mean GCS on Day 4-10. Neither CXCL-16 nor FABP3 showed a significant correlation with DCI.

Table 2. *Variables associated with CXCL-16 and FABP3 in cross-sectional analysis.*

Parameter	CXCL-16	FABP3
mFischer 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

Values are Spearman correlation coefficients (rho). ∗P<0.05, ∗∗P<0.001. CRP, C-reactive protein; mFischer score, modified Fischer score; WFNS, World Federation of Neurosurgical Societies score; CXCL-16, Chemokine (C-X-C motif) ligand 16; FABP3, Fatty-acid binding protein; NLR, neutrophil-lmphocyte ratio; GCS, Glasgow coma scale

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. Table 1 showed that the variables associated with unfavorable outcome at Day 30 included mFischer 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 30-day outcome (Table 3).

Discussion

In the present study, we observed an association between elevated serum level of both CXCL-16 and

Table 3. *Multivariate logistic regression analyses for variables associated with unfavorable outcome (mRS>2) on day 30 in patients with aneurysmal subarachnoid hemorrhage.*

	unfavorable outcome at Day 30			
Variables	B	Odds ratio	95% CI	p-value
CXCL-16 level> cutoff	-2.243	5.945	0.018-0.644	***0.015***
creatinine	0.048	2.647	0.990-1.111	0.104
mFisher score	0.474	0.515	0.440-5.860	0.473
WFNS score	0.418	0.988	0.666-3.463	0.320
CRP	0.009	0.943	0.991-1.027	0.331
FABP3 level> cutoff	-1.000	0.910	0.047-2.871	0.340
age	0.016	0.198	0.946-1.093	0.656

CRP, C-reactive protein; mFischer score, modified Fischer 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

FABP3 and an unfavorable outcome at Day 30 after aSAH. Besides, serum CXCL-16 level with a cut-off level of > 446.7 pg/mL was identified as an independent predictor of an unfavorable outcome.

SAH cause brain injury via glutamate excitotoxicity, leading to an excessive Ca^{2+} influx and can induce an apoptotic cascade.[16] Wang et al. found that CSF levels of glutamate are significantly higher in patients with severe SAH (WFNS grade 4-5 and mFischer score 3-4) than those with less severe SAH (WFNS grade 1-3 and mFischer score 1-2).[17] CXCL-16 can promote physiological neuroprotective mechanisms that counteract neuronal cell death due to ischemic and excitotoxic insults mediated by glutamate.[18] CXCL-16 acts directly on astrocytes to release soluble factors essential to mediate neuroprotection against excitotoxic damage due to excessive glutamate exposure.[19] 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.[20] 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[21] and in acute coronary syndrome.[22] There may be several explanations for these results: (i) CXCL-16 activates CD8+ T cells, leading to apoptosis in the surrounding of an atherosclerotic plaque;[23] (ii) CXCL-16 has the ability to direct the migration of activated T lymphocytes to the lesion tissue; (iii) where it can promote plaque formation and thrombosis by locally secreting multiple cytokines and matrix metalloproteinases.[24] Both apoptosis[25-26] and damaged cerebral microvasculature[27] 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.[28] Similar to TNFSF14, which has recently been emerged as a novel marker of the outcome in aSAH, CXCL-16 is a "Janus" faced molecule exerting both pro- and anti-inflammatory effects.[15]

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 mFischer), CRP and NLR, as well as the serum level of CXCL-16, while negatively correlated with serum creatinine, platelet count and GCS. In accordance, *Yilman et al.* found that serum level of FABP3 showed significant association with Hunt Hess score and Fischer score in a small cohort of patient with SAH.[27] In a study by *Zanier et al.*, the CSF level of FABP3 showed significantly higher peak levels in patients with severe SAH than mild SAH patients.[8] A study by *Wunderlich et al.* 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.[6] Importantly, FABP3 is a more sensitive marker for minor brain injury than the commonly used markers such as S100B or NSE.[4] Moreover, it has an excellent ability to differentiate between CT-positive and CT-negative mild TBI patients.[29] FABP3 is also known to be a sensitive marker in acute coronary events and cardiac failure.[30-32] 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.[33] Its serum concentration remains increased for days after ischemic stroke;[6] 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].[34] FABP3 can bind the 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.[34]

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.[6,29] 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. Whether these findings are epiphenomenal or causal in developing of aSAH require further studies involving other known prognostic markers. Considering that both markers are key players in different pathological mechanisms (FABP3 is a damage marker, while CXCL-16 rather reflects the stage of vascular inflammation), their observed correlation in our study deserves further explorations.

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.[35] 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 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.

A paradigm shift is currently going on from testing single picked markers towards high throughput genomics or proteomics identifying biomarker signatures in this field. These would more likely result in a comprehensive identification and mapping of entire biological pathways causally involved in SAH pathogenesis, and complications such as DCI. Based on these findings, a personalized therapeutic intervention can ideally be guided leading to a favourable outcome in patients with aSAH. Beside conventional CNS specific markers (e.g. S100B, GFAP etc), novel molecules should be involved to create clusters of patients with different risk for complications and adverse events. Indeed, a deeper insight of the pathophysiological roles of such markers will be needed in the future. In our view, a multiple-marker based approach will emerge as a future perspective to provide a personalized care for patients with aSAH. By the analogy of precision oncology, disease and risk specific markers should be individually determined and processed by arteficial intelligence (AI) in networks supporting clinical decisions that may result in a favourable clinical outcome.

Limitations

First, our study sample was relatively small, and the duration of follow-up was short. Results need to be confirmed on a larger sample size of confirmed aSAH. Secondly, sampling at multiple time points instead of a single shot could clarify whether the kinetics of CXCL-16 and FABP3 differ in various subgroups of aSAH patients. Thirdly, the rigid inclusion/exclusion criteria limit the generalizability of the study.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors declare no conflict of interests.

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