Biomarkers for prediction of cerebrovascular diseases associated complications and outcome

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THÉSE

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Le Doyen

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".

A mi familia,

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Abstract

Stroke is a medical emergency that takes place when the supply of blood to an area of the brain is stopped or reduced. Brain injury produced by the initial cerebrovascular accident is responsible of a high proportion of associated deaths (5.9 million annually); however complications occurring in the days following the initial accident will be also responsible of important rates of morbidity and mortality. Post-stroke infections, more concretely pneumonia and urinary tract infections are the most common post-stroke complication, developing in 23-65% of the patients and producing around 30% of the mortality occurring in the acute phase of stroke. Even if their early diagnosis is necessary to start with antibiotherapy in the shortest delay, this is a challenging task for the doctors as there are not well established criteria between the different studies.

Consequently, in order to apply new treatment strategies as soon as possible and to improve patient's outcome, the aim of the present study was to discover and validate infection biomarkers able to predict the patients that will develop an infection during their hospital stay.

By using quantitative proteomic approaches and including data of the literature, we highlighted two potential promising risk stratificator markers: neopterin and Serum Amyloid A (SAA). After validating the results with immunoassays on larger multicentric cohorts, we showed that neopterin correlated correctly with infection development from three days after hospital admission. More interestingly, SAA presented also this correlation capacity, but in this case it was able to distinguish at hospital admission the two groups of patients. Afterwards, using a home-made Panelomix tool, we combined neopterin and SAA with other individual clinical parameters (WFNS, GCS, age, WBC) in order to improve their prediction capacity. The combination formed by SAA, WFNS, WBC and age improved importantly the specificity (SP) and sensitivity (SE) values when comparing with individual markers, obtaining at hospital admission 100% SP for 64.3% SE.

In conclusion, this work has demonstrated that SAA and/or SAA in combination with other markers is an excellent tool to start antibiotherapy in an earlier stage, leading to a better management of the patients and to an improvement of their associated outcomes.

Résumé

Un accident cerebrovasculaire (AVC) est une urgence médicale qui se produit lorsque l'approvisionnement en sang à une zone du cerveau est arrêté ou réduit. Les lésions cérébrales

produites par l'accident vasculaire cérébral vont être responsables d'une forte proportion de décès (5,9 millions par an), mais les complications qui surviennent dans les jours qui suivent l'accident initial seront également responsables d'importants taux de morbidité et de mortalité. Les infections et plus concrètement la pneumonie et les infections des voies urinaires, sont la complication la plus fréquente, se développant chez 23-65% des patients et produisant environ 30% de decès. Même si leur diagnostic précoce est nécessaire pour commencer avec l'antibiothérapie dans le plus court délai, celle-ci est une tâche difficile pour les médecins car il n'existe pas de critères bien établis entre les différentes études.

Par conséquent, afin d'appliquer de nouvelles stratégies de traitement et d'améliorer le pronostic du patient, l'objective de cette étude était de découvrir et de valider des biomarqueurs d'infection capables de prédire les patients qui développeront une infection pendant leur hospitalisation.

En utilisant des approches de protéomique quantitative et en incluant des données de la littérature, nous avons mis en évidence deux potentiels biomarqueurs: la néoptérine et la Sérum Amyloide A (SAA). Après avoir validé les résultats sur des cohortes multicentriques plus larges, nous avons montré que depuis trois jours après l'admission à l'hôpital la néoptérine corrèle correctement avec le développement de l'infection. D'une manière encore plus intéressante, la SAA a aussi présenté cette capacité de corrélation mais d'une manière plus précoce, étant déjà significativement différentiel à l'admission à l'hôpital. Ensuite, à l'aide d'un outil fait maison, Panelomix, nous avons combiné la néoptérine et la SAA avec d'autres paramètres cliniques (WFNS, GCS, âge, GB) afin d'améliorer leur capacité de prédiction. La combinaison SAA, WFNS, GB et âge améliore les valeurs de spécificité (SP) et de sensibilité (SE) lors de la comparaison avec les marqueurs individuels, obtenant à l'admission à l'hôpital 100% de SP pour 64,3% de SE.

Les résultats actuels ont montré que la SAA et /ou la SAA en combinaison avec d'autres marqueurs est un excellent outil pour commencer avec l'antibiothérapie à un stade plus précoce, ce qui conduira à une meilleure prise en charge des patients et à une amélioration de leurs pronostic.

Abbreviations

2D-gel: two dimensional gel electrophoresis

aSAH: aneurysmal subarachnoid hemorrhage

AD: Alzheimer disease

AUC: area under the ROC curve

A2DS2: age, atrial fibrillation, dysphagia, sex, stroke severity

CDC: centers for disease control and prevention

CHAPS: 3 [(3cholamidopropyl) dimethylammonio]-1-propanesulfonate

CI: confidence interval

CID: collision-induced dissociation

CRP: C-reactive protein

CSF: cerebrospinal fluid

CT: computed tomography

DAMPS: damage associated molecular patterns

DCI: delayed cerebral ischemia

DND: delayed neurological deficit

ELISA: enzyme-linked immunosorbent assay

ECG: electrocardiogram

EVD: external ventricular drain

ETD: electron-transfer dissociation

FDR: false discovery rate

FLAIR: fluid attenuation inversion recovery

FTICR: fourier-transform ion cyclotron resonance

GCS: glasgow coma scale

GFAP: glial fibrillary acidic protein

GOS: glasgow outcome scale

GTP: guanosin-5-triphosphate

HCD: higher-energy collisional dissociation

HPLC: high-performance liquid chromatography

HRP: horseradish peroxidase

IAA: 2-iodoacetamide

ICAT: isotope-coded affinity tags

ICBT: iterative combination of biomarkers and thresholds

ICH: intracerebral hemorrhage

ICU: intensive care unit

IFN-γ: interferon-γ

iTRAQ: isobaric tags for relative and absolute quantification

IL: interleukin

IPG: immobilized pH gradient

IQR: interquartile range

ISAT: international subarachnoid aneurysmal trial

IT: ion trap

MRI: magnetic resonance imaging

MRM: multiple reaction monitoring

mRS: modified Rankin scale

MSD: mesoscale discovery

NIHSS: national institutes of health stroke scale

NK: natural killer

NSE: neuron specific enolase

NP: neopterin

PCT: procalcitonin

PD: Parkinson disease

pI: isoelectric point

POCT: point-of-care testing

PRM: parallel reaction monitoring

Q: quadrupole

RBC: red blood cells

ROC: receiver operating characteristic

ROS: reactive oxygen species

rtPA: recombinant tissue plasminogen activator

SDS: sodium dodecyl sulfate

S100β: S100 calcium binding protein β

SAA1/2: serum amyloid 1/2

SE: sensitivity

SILAC: stable isotope labeling with amino acids in cell culture

SMARTER: simultaneous marker discovery and verification for the rapid translation of exogenous reference material

SIRS: systemic inflammatory response

SP: specificity

SRM: selected reaction monitoring

TIA: transient ischemic attack

TOF: time of flight

OGE: off-gel electrophoresis

OT: orbitrap

TEAB: tetraethylammonium bromide

TCEP: tris-(2-carboxyethil) phosphine hydrochloride

TMT: tandem mass tags

TOAST: trial of org 1072 in acute stroke treatment

UTI: urinary tract infection

WB: western blot

WBC: white blood cells

WFNS: world federation of neurosurgical societies

Chapter I

Introduction

1. Stroke overview

Stroke is the leading cause of disability and the second cause of mortality worldwide. In 2013 about 10.3 million people had a stroke. However, the incidence has strong variations according to the geographical location (1, 2). In France for example, the incidence ranges around 240 per 100000 people while in Russia it can be around 600 per 100000 people (3). The proportion of deaths produced by cerebrovascular accidents worldwide is 9%, causing 5-7 deaths per million people per year (4, 5). Due to the thorough healthcare applied during the last decades, the stroke mortality is decreasing faster than the stroke incidence, which leads to an increase in the number of survivors and to a need of health-care patient management improvement (6).

There are two different types of cerebrovascular accident: ischemic stroke, which accounts for 87% of all strokes, and hemorrhagic stroke which accounts for the remaining 13% (3). In both cases, due to the lack of glucose and oxygen, brain cells start to die producing the associated symptomatology and complications. Transient ischemic attacks (TIA) are a condition, similar to stroke, in which the blood flow of the brain is blocked for a short time. However, in this case the brain cell damage is not permanent and cells do not die, so symptoms will resolve in less than 24 hours (7).

In this thesis project we will use the terms cerebrovascular accident or stroke indistinctly to refer to both types of stroke (ischemic and hemorrhagic). The terms “ischemic stroke” or “hemorrhagic stroke” will be used respectively to refer to each of the subtypes.

1.1. Ischemic stroke

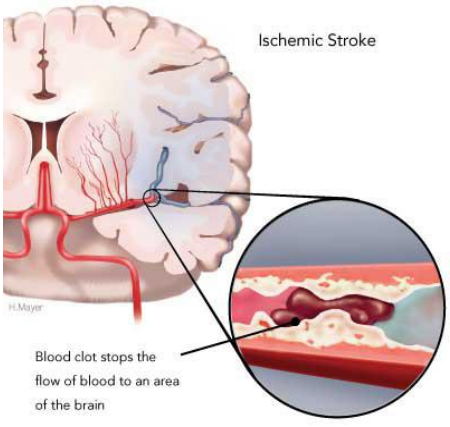

Figure 1: representation of an ischemic stroke with the blood clot stopping the blood flow.

Ischemic stroke occurs when an artery that supplies oxygen-rich blood to the brain is blocked (Figure 1) (3, 8). The incidence increases importantly with age, occurring 95% of ischemic strokes in people older than. High blood pressure and atrial fibrillation as well as high cholesterol levels, diabetes mellitus, cigarette smoking, alcohol consumption and obesity are some of the main modifiable factors that increase the risk of stroke development (9-12). Following a healthy lifestyle however, can reduce this risk improving the rates of death.

According to the TOAST classification, able to identify the mechanism responsible of vessel occlusion, different subgroups of ischemic stroke can be defined (13, 14). Thrombotic stroke occurs when the blood clot is formed inside one of the brain arteries (15). Clinically, it accounts for around 50% of all the ischemic strokes and in most of the cases it is due to disease of the arterial wall, such as atherosclerosis. Depending on the location of the blockage, two different subtypes can be distinguished: large vessel thrombosis, when the blockage of large arteries is produced or lacunar stroke, that occurs when a small artery arising from larger arteries of the brain is blocked (16). This subtype of ischemic stroke has usually a better outcome than the first subtype, as the brain damage is usually smaller than in the blockage of large arteries. Cardioembolic stroke is another subgroup of stroke also caused by a clot that blocks an artery, however in this case, the clot will be formed somewhere else than in brain. Cardiac diseases will be usually the responsible of this subtype of stroke. One cardiac cause must be at least identified, being in most of the cases atrial fibrillation the responsible one. This will account for 25-35% of all ischemic strokes (3).

1.1.1. Neurological evaluation and diagnosis

Once the stroke has been developed and the patients are admitted to the hospital, a physical examination and clinical diagnosis are key to define the type of stroke occurred, the associated severity and the required treatment. The National Institutes of Health Stroke Scale (NIHSS) is one of the most objective and used classifications to evaluate the impact of the cerebrovascular accident and the neurological state of the patients. It is composed by 11 items that evaluate the level of consciousness, the eye movement (17), the visual field, grade of facial paralysis, arm and leg movement, coordination, sensory, language, speech and patient inattention. For each of the symptoms, when the evaluation is normal, a score of 0 will be attributed, while when the evaluation is altered a maximum score of 4 can be attributed to each item. The maximum possible score is 42, with which patients are in a very severe condition (Table 1).

Neurological assesment			Outcome assesment		
Scale	**Score**	**Criteria**	**Scale**	**Score**	**Criteria**
NIHSS	0	No stroke symptoms	**Modified Rankin score**	0	No symptoms
	1-4	Minor stroke		1	No significant disability
	5-15	Moderate stroke		2	Slight disability
	16-20	Moderate to severe stroke		3	Moderate disability
	21-42	Severe stroke		4	Moderately severe disability
				5	Severe disability
				6	Dead

Table 1: description of NIHSS and Modified Rankin Scale.

If the total score changes at least 4 points a neurological changement in patient state will be considered; more concretely, an improvement will be observed if it produces a decrease in the total score or a worsening if it produces an increase on it (18). Similarly, to evaluate the impact that the stroke will produce in patient's long-term outcome and to decide about the best practice to follow, a different scale is available. The modified Rankin Scale (mRS) is a clinical scale used to evaluate patient's disability degree after suffering a stroke. It is composed by 6 items that runs from perfect health, this is, no symptoms to death. At present, it is the most used outcome scale in stroke clinical trials (Table 1) **(19)**.

However, apart from this initial neurological evaluation, to correctly make the diagnosis step signs and symptoms need to be also evaluated. Usually they develop very quickly after the onset, but depending on the type of ischemic stroke and on the region of the brain that has been affected, clinical presentations can vary. Even though, common symptoms may include: sudden weakness of face or of a part of the body (arms, legs), confusion and sudden vision and coordination troubles (20).

Patients presenting these symptoms will be submitted to imaging analysis to exclude other types of cerebral lesions (stroke mimics) and to distinguish the ischemic stroke from the hemorrhagic one. The most common used modality is the computed tomography (CT) scanning as it is easy, fast, not expensive and quite sensitive to detect acute hemorrhages as well as brain masses. However, if the stroke is small or it is an acute ischemic stroke, CT scans will not be sensitive enough to detect it. The resolution of magnetic resonance imaging (MRI) is better than the resolution of CT scans: they are able to detect acute ischemic strokes and in around half of the transient ischemic attack patients (TIA), this is in patients with a transient episode of neurologic dysfunction produced by ischemia, it will show ischemic lesions (21). Furthermore, for the detection of intracerebral hemorrhages MRI is as sensitive as CT. The main drawback is that this kind of scanners are more expensive than CT scans and so less available. Nevertheless, in stroke patients needing a diagnosis, MRI will be performed if possible unless this produces a delay in the analysis time, in which case the CT should be performed (22-24).

1.1.2. Patient management

After diagnosing the type of the stroke, the goal for the acute management is to stabilize the patient and to prevent the complications that can worsen the outcome.

Because thrombus is the most common cause of ischemic stroke, the first practice is the dissolution of blood clots by intravenous fibrinolytic therapy (rtPA), one of the most effective

treatments and the only FDA-approved drug. rtPA is a fibrin specific thrombolytic agent that under the action of plasminogen produces plasmin, a protease able to cleave the clot (25). Once the thrombus is broken, the circulation of the affected brain region is restored and the associated injury reduced. According to the expert group recommendations, the time window for the administration of this therapy is decreased to the first 4.5 to 6 hours following the onset, improving the efficacy when this time window is reduced (26).

However, it must be considered that some of the complications occurring after stroke are related to this rtPA administration. Recanalization is achieved only in 50% of the cases and in those cases in which it has been possible to perform it, important complications can be found; for example, in more than 34% of the cases a reocclusion of the recanalized artery is documented (27). Furthermore, in about 6-7% of the patients treated with rtPA an intracerebral hemorrhage can appear as the main adverse effect (28). Nevertheless, data studying this complication have evidenced that most of the patients developing an intracerebral hemorrhage had received the canalizations after 6 hours after the symptom onset. Therefore, the time between the symptom onset and the hospital admission is trying to be reduced by improving the early recognition of stroke (29).

Infections occurring in the subacute phase of stroke have also become one the most important post-stroke complications. They have a prevalence of 30% and more importantly they account for one third of all the stroke associated deaths. In this thesis project we have focus our research attention on this complication, postulating that the research of markers able to predict its development could importantly help in the decision making process and outcome of the patients. More detailed information will be explained in the section: “Post-stroke inflammation and infection biomarkers overview” of this introduction.

Brain edema is other common complication occurring after stroke. The ischemic event that alters the neurovascular integrity, reduces the permeability of the blood brain barrier and increases the pass of different fluids. This results in a vasogenic edema that increases the rates of death. Therefore, normotension and normothermia should be maintained in order to avoid its development.

Additionally to all of these complications, it has been documented that stroke and ischemic heart disease share some of their most important risk factors, being common in those patients that have suffered from an ischemic stroke to develop a heart attack. Furthermore, autonomic control alterations produced by stroke will predispose the patients to cardiac complications. Consequently, ECG evaluation should be performed in all the stroke patients. If alterations or

modifications are found continuous monitoring of the cardiac activity should be done for at least 3 days.

1.2. **Hemorrhagic stroke**

Hemorrhagic stroke are the second subtype of stroke accounting for around 13% of all the cerebrovascular accidents and producing for about 40% of the deaths. They can be divided in: intracerebral hemorrhage or subarachnoid hemorrhage. In both cases, as a consequence of blood vessels alteration, brain artery ruptures producing intracranial bleeding (30).

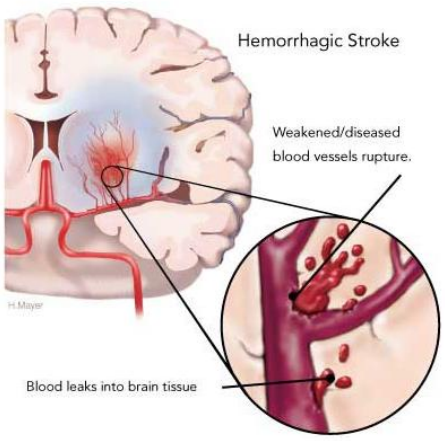

Figure 2: representation of a hemorrhagic stroke.

Intracerebral hemorrhage

Intracerebral hemorrhage (ICH) is a type of hemorrhagic stroke produced by the presence of blood within the ventricles or the brain tissue. It accounts for around 10-20% of all strokes and it is considered a medical emergency because many of the cases are followed by a rapid clinical deterioration and death (31) (Figure 2).

The symptoms associated to ICH can vary depending of the region of the brain that has been affected. Nevertheless, a severe headache and vomiting are some of the most characteristics ones. Bleeding from the ear or coma can also be present (30). CT scan and MRI are the two methods of choice when ICH is suspected (22).

Once the cause of the bleeding has been set, different types of treatments can be applied. First of all, blood pressure should be maintained at normal levels to decrease the probability of bleeding. Intracranial pressure should also be controlled to reduce the risk of poor outcome. For this maintenance, the removal of CSF from the ventricles is usually performed.

Surgery, able to remove important volumes of blood is recommended in those patients with a hematoma greater than 3cm. A craniotomy or stereotactic aspiration is performed depending on the location of the hemorrhage. Complications after ICH are common including pneumonia, respiratory failure and sepsis. A special care for their prevention should be applied (31). Therefore high blood pressure and amyloidosis, the two main risk factors associated with ICH, should be controlled. Diabetes mellitus, smoking, alcohol and menopause also increase the risk of ICH development(32).

Subarachnoid hemorrhage

Subarachnoid hemorrhage (SAH) is a subtype of stroke produced by bleeding in the subarachnoid space, this is the space located between the brain and the tissue around the brain. It accounts for only 1-7% of all strokes, but because the disease affects young subjects and the associated mortality and morbidity is very high, the rapid diagnosis and management are crucial in clinical practice (33).

The incidence of SAH changes a lot depending on the regions of the world, being the intermediate one 9.1 (95% CI 8.8 to 9.5) people per 100000 annually. Much more cases are found in Finland 19.7 (95% CI 18.1 to 21.3) and Japan 22.7 (95% CI 21.9 to 23.5) and little bit less cases in South or Central America. The incidence in Finland and Japan was 2.2 (95% CI 2.0 to 2.4) and 2.5 (95% CI 2.4 to 2.6) times higher than in the reference group and 2.2 (95% CI 1.6 to 2.9) times lower in South or Central America (34, 35). Several factors such as genetics, different mean age among populations or better case findings could explain these variations, however for instance they remain only speculations.

The mean age of patients suffering from SAH is between 45 and 55, increasing the incidence importantly with age (33). Among the people of the same age, women are more prone to develop a SAH than men (35). However, genetics and more importantly, modifiable lifestyle factors as smoking, hypertension and alcohol consumption could be described as the main factors contributing to its development (36).

In most of the cases (85%) it is produced by the rupture of a cerebral aneurysm (aSAH) (37). However, non-aneurysmal perimesencephalic hemorrhage, arterial dissection, cerebral arteriovenous malformation and dural arteriovenous fistula causes the resting 15%.

The first month following aSAH is the most critical one, as half of the patients will die during this period. 40% of the patients will die within the first week following the initial hemorrhage: between 15-30% of them will die before arriving to the hospital and another 25% will die within the first 24 hours (38, 39). Among the survivors, a vast majority will remain with important motor and neurological deficits (40).

1.2.1. Neurological evaluation and diagnosis

Similarly as performed in ischemic stroke patients, a neurological evaluation of aSAH patient's state is needed at hospital admission. Specific SAH clinical scales used to perform this categorization are represented in Table 2. These scales are useful to standardize the

management among different centers and to evaluate the effect of different treatments (Table 2). The most used ones are: the Fisher grade, the Hunt and Hess, the Glasgow Comas Scale (GCS) and the World Federation of Neurosurgeons (WFNS), a scale that uses the Glasgow coma score and the focal neurological deficit to evaluate the severity of the hemorrhage.

Scale	Score	Criteria		Subgroups
Fisher		**Amount of hemorrhage**		
	1	No blood		Small hemorrhage
	2	Diffuse depositon or thin layer. No clots		
	3	Dense collection of blood		High hemorrhage
	4	Intracerebral or intraventricular clots		
Hunt and Hess	0	Unruptured aneurysm		
	1	Asymptomatic, or minimal headache		Good neurological state
	2	Moderate to severe headache		
	3	Confusion, drowsiness		
	4	Moderate to severe hemiparesis		Poor neurological state
	5	Deep coma		
GCS		**Eyes opening**		
	1	Never		
	2	To pain		
	3	To speech		
	4	Spontaneusly		
		Verbal response		
	1	None		GCS 3-8 : Severe brain injury
	2	In comprehensible sounds		
	3	Inappropiate words		GCS 9-12: Moderate brain injury
	4	Confused		
	5	Oriented		GCS 13-15: Mild brain injury
		Motor response		
	1	None		
	2	Extension to pain		
	3	Abnormal		
	4	Withdrawal		
	5	Localized pain		
	6	Obeys commands		
WFNS		**GCS**	**Motor deficit**	
	1	15	Absent	Good neurological state
	2	14-13	Absent	
	3	14-13	Present	Poor neurological state
	4	12-7	Present or absent	
	5	6-3	Present or absent	

Table 2: description of different clinical scales used to evaluate the neurological state of aSAH patients.

To evaluate patient's long term outcome a different scale is also available: the Glasgow Outcome Scale, a scale that evaluates the outcome and recovery of the patients after aSAH. In this case, it is not performed at hospital admission but 3 months after the symptom onset (GOS 3), six months after (GOS 6) or one year after (GOS 12). In patients with subarachnoid hemorrhage it

has been used to evaluate their recovery in five different categories. Dead, Vegetative State, Severe Disability, Moderate Disability or Good Recovery (Table 3).

Scale	Score	Criteria	Subgroups
GOS	1	Death	Poor outcome
	2	Vegetative: coma or severe deficit	
	3	Severe disability: significant neurological deficit that interferes with daily activities	
	4	Moderate disability: minor neurological deficit that does not interfere with daily activities	Good outcome
	5	Good recovery: returned to the original functional level	

Table 3: description of GOS, a clinical scale used to evaluate the outcome of the aSAH patients.

Apart from the neurological evaluation, the clinical presentation of the patients must be also evaluated at hospital admission. In one third of the patients the only characteristic symptom occurring after aSAH is a severe and sudden headache described as “the worst ever”. Nevertheless, it is not the severity, but the suddenness of onset, the characteristic feature of aSAH. Less severe headache can also be present between 2 to 8 weeks before the hemorrhagic rupture onset in patients with rupture of small cerebral aneurysms (41). Seizures, will be present in 1 out of 14 patients and are not characteristic of other non-hemorrhagic severe headaches, so its presence could be a strong indicator of subarachnoid hemorrhage (42, 43). Vomiting, confusion, coma and neck stiffness are present in an important number of patients suggesting the presence of aSAH (44). While, the sudden headache is the main characteristic feature of subarachnoid hemorrhage, only one in ten patients presenting with severe headache will be diagnosed with aSAH. Among these diagnosed patients, half of them will be neurologically intact. Therefore, apart from the physical examination, diagnostic tests are necessary.

The first method of choice for diagnosing aSAH is the CT scanning without contrast. It is a method with high sensitivity when performing in the first 12 hours following the hemorrhage, identifying 98-100% of affected patients (45). This sensitivity is dependent on the resolution of the scanner, on the severity of the hemorrhage, and on the time between the symptom onset and clinical examination; CT is extremely sensitive during the first 24 hours but it diminish importantly from this moment (23). Consequently, depending on these factors, clinicians can found high number of false negative cases in those patients suffering from aSAH with neurologically normal scan. Therefore, when a patient has suffered from a thunderclap and the CT is normal, other diagnostic tools should be performed. Any patient with normal CT scan but with suspected SAH might require a lumbar puncture to analyze the cerebrospinal fluid (46, 47). The ideal time to perform it is around six hours after the severe headache (preferably 12 hours), in order to give enough time to hemoglobin to convert into oxyhemoglobin and/or bilirubin. The

presence of oxyhemoglobin can be the result of a traumatic tap, not confirming the aneurysmal rupture, while the presence of bilirubin, only synthetized *in vivo*, will confirm the diagnosis of aSAH. This method will allow confirming the presence of aSAH in a small number of patients not detected with the CT scan (23, 48). MRI is also a useful tool to diagnose aSAH when there are several days between the headache onset and the diagnostic analysis. The sensitivity of CT decreases progressively and the MRI is a better method to detect blood. The most accurate techniques are fluid attenuation inversion recovery (FLAIR) and T2-star images (23, 45).

1.2.2. Patient management

The management of patients with subarachnoid hemorrhage has three different phases: the first and most important one is the stabilization of the patient at hospital admission: the aggressive effect of the hemorrhage is the main responsible of patient's poor outcome. Therefore, resuscitation of patients arriving at the hospital with a reduced level of consciousness or with a focal deficit is mandatory in order to improve their long-term state.

Afterwards, it is necessary to treat the aneurysmal rupture and finally to avoid the complications responsible of important number of mortality and morbidity.

Reparation of the aneurysmal rupture is mandatory to avoid rebleeding, a dangerous and modifiable complication responsible of the highest rates of mortality and morbidity occurring after aSAH. During the first 72 hours following the initial hemorrhage between 8-23% of patients will develop it. The most used therapeutic options to avoid this complication are surgical clipping or endovascular coiling of the affected vessel. Surgical clipping is a procedure in which a craniotomy will be followed by the implementation of a titanium clip around the neck of the aneurysm, while endovascular coiling is a medical device used to occlude an aneurysm (49, 50). For this purpose a platinum microcatheter is inserted into the femoral artery to reach the affected cerebral aneurysm. Afterwards, Guglielmi detachable coils coming from the microcatheter are released into the aneurysm to densely block it (Figure 3). The choice between the two methods depends on several factors such as: the aneurysm location, aneurysm size, patient's age and/or patient's condition. Many aneurysms are not equally adapted for one of the techniques, being one more suitable than the other. Nevertheless, for those patients in whom both methods are suitable, coiling is preferred (23). According to the International Subarachnoid Aneurysmal Trial (ISAT) – the gold standard study of this subject – endovascular coiling will provide a better one year recovery and prognosis than surgical clipping (38, 51).

Patients who survive to the initial hemorrhage and have the aneurysm repaired, are also at risk of important complications that could worsen their outcome. Delayed cerebral ischemia (DCI) is

the most important cause of death and morbidity in patients who survive to the initial bleeding (52). One third of the hospitalized patients will develop a DCI, a neurological deterioration attributed to an ischemic event. It will develop within the first two weeks following aSAH and will be responsible of a decrease in the level of consciousness. Physiological mechanisms underlying DCI are still unclear. Nevertheless, early brain injury produced by the hemorrhage is one of the most accepted ones: neurons and endothelial cell death, the release of inflammatory and vasoactive factors or cortical spreading depression among others could importantly contribute to arteries ischemia. Historically, angiographic vasospasm, (narrowing of cerebral arteries) was proposed as the main responsible of tissue ischemia. The process was thought to be activated by the presence of blood metabolites in the subarachnoid space (Figure 4) (53-56).

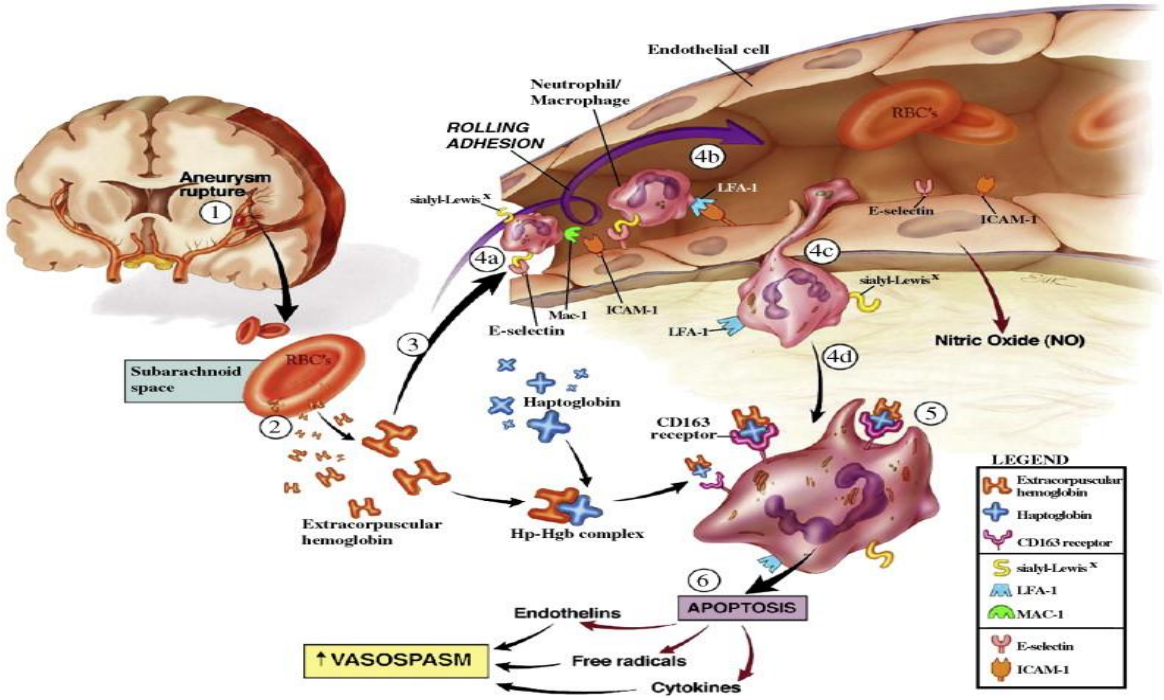

Figure 4: cascade of events happening after the rupture of a cerebral aneurysm and leading to the development of vasospasm

However, at present there is increasing evidence that the DCI has a multifactorial etiology, and that it is not only produced by the diminution of arteries flow. The angiographic visualization of cerebral vessel constriction altogether with clinically proven deterioration, has produced in the literature a mix of these two concepts, using the word “vasospasm” to describe not only the narrowing of cerebral vessels but also the ischemia produced in cerebral arteries (57).

For instance, there are no methods to prevent the development of vasospasm and DCI, but there are different proposed treatments (58). Euvolemic induced hypertension is recommended in the DCI therapy as it is able to ameliorate the ischemia, increasing the brain oxygenation and

reducing the neurological deficits produced by the DCI. Another widely used treatment is the endovascular therapy. Even if its effect on outcome is controversial, its administration is recommended in those patients that do not respond to hypertensive therapy (23, 31, 59). Calcium channel antagonists as nimodipine have been also widely used to prevent the vasospasm. Nevertheless, and even if at present it has been shown that they are not able to revert the vasospasm incidence, they improve patient's outcome and their administration on all aSAH patients is recommended (23, 60).

Hydrocephalus is another complication produced by an abnormal accumulation of CSF in the brain, which leads to an enlargement of cerebral ventricles. It is a common complication developing in 20-30% of the patients and leading to a poor outcome. External ventricular drainage or lumbar drainage are the possibilities for the management of patients suffering from hydrocephalus. Patients admitted to the hospital with a severe brain injury should require a continuous intracranial pressure monitoring (61, 62).

Although rebleeding, DCI and hydrocephalus are usually the focus of research in aSAH, nosocomial infections have been reported to have deleterious effects on outcome of the patients. As previously explained for ischemic stroke patients, we have focus our attention on this complication to try to better manage the patients and prevent its development. During this thesis project, the main research work has been performed in patients suffering from aSAH, as for the instance it remains a real unknown complication in these group of patients. In the next section of this introduction we will explain the molecular mechanisms leading to nosocomial infections as well as the state of the art in biomarker discovery.

2. Post-stroke inflammation and infection biomarker overview

According to the Biomarkers Definitions Working Group, a biological marker or biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal and pathological biological processes, or pharmacological responses to a therapeutic intervention (63).

The use of biomarkers for the diagnosis and management of several diseases has become so essential that their presence in clinical routine is now widely accepted (64), obtaining almost one specific biomarker for each biological disorder (65, 66). In stroke associated biomarkers, several studies have been performed to discover molecules able to identify the different types of stroke (ischemic, hemorrhagic and mimic), to study the etiology or the severity of the stroke, as well as to identify patients who need specific therapies such as antibiotics in case of infection (67-70).

2.1. **Inflammation biomarkers**

The study of inflammation and inflammatory markers has been also a field of high interest (71, 72), as it is one of the most important physiopathological mechanisms occurring after a general brain injury and leading to a poor outcome (73). Furthermore, it has been proposed as the main responsible of some important complications occurring after ischemic stroke and subarachnoid hemorrhage such as ischemic events (DCI) or infection more concretely (74).

During the occlusion of the brain vessels, the deprivation of oxygen and glucose produces the death of the localized cells. These cells will release damage associated molecular patterns (DAMPS), that will produce the activation of the inflammatory cascade, increasing the local production of molecular (cytokines, chemokines, matrix metalloproteinases and adhesion molecules) and cellular markers (leukocytes, microglia and acute phase reactants).

Cytokines are a family of small proteins important in cell activation, proliferation and differentiation. Under normal conditions, their receptors are very low abundant and cytokines become almost undetectable. Nevertheless, under the action of a cerebrovascular accident or an important brain injury, they are extremely upregulated (75). In plasma samples of patients suffering from aSAH, cytokine levels were found to peak in the first 24 hours following the hemorrhage (75). Proinflammatory IL-1β, TNF, IL-6 and anti-inflammatory TGF- β and IL-10 have been the most studied cytokines in brain ischemia. Their higher levels in blood correlate with poor outcome, as they are supposed to exacerbate the brain injury (76). Concentrations of IL-6 increase also continuously during the first 24 hours after the brain injury, correlating higher levels with higher risk of in hospital death (77).

Chemokines are a group of small cytokines highly involved in inflammatory and immune response. In the last years, an important number of studies have shown that different types of chemokines as MCP-1 or MIP-1α are overexpressed in animal models presenting with an ischemic stroke or SAH. Accordingly, their inhibition or decrease has shown a reduced level of injury (76, 78).

Matrix metalloproteinases are another group of molecules contributing to the neuroinflammatory response. After an ischemic stroke, up-regulation of MMP-9 concentrations is correlated with neurological impairment and hemorrhagic transformation (79) (80).

Adhesion molecules are responsible of Interactions produced between leukocytes and the endothelium. Blood concentrations of ICAM-1 and VCAM-1 have been shown to be increased in patients with ischemic and hemorrhagic stroke (81). Different studies conclude that during the

stroke event adhesion molecules have an important role in leukocyte infiltration and in neuronal secondary lesion (76, 82) .

Concerning the cellular components, leukocytes and microglia are the most widely activated and accumulated cells after a cerebrovascular event. Leukocytes that will participate in host defense are increased in patients with acute ischemic stroke and subarachnoid hemorrhage (83, 84). Microglia account for 5-20% of CNS glial population. Animal studies have shown that under a brain injury insult, macrophages and microglia will produce cytokines as IL-1 and TNF-α (76).

Acute phase response includes a number of important changes produced in response to brain injury. In stroke patients, the most studied acute phase markers are C-reactive protein, serum amyloid A, and fibrinogen. CRP plasma concentrations in patients suffering from ischemic stroke or subarachnoid hemorrhage are significantly elevated when comparing with control subjects. Its levels correlate also with the severity of the lesion and with the prognosis of the patients, showing higher concentrations in those patients with high lesion and poor outcome (85). Similarly, serum amyloid A and fibrinogen, less studied molecules have also been correlated with stroke severity and prognosis (86).

2.2. **Post-stroke infection**

All the previously described inflammatory events produce important cell damages after stroke. However, it has become increasingly evident that systemic immune suppression, occurring as a compensatory mechanism against inflammation and brain damage, is also responsible of important complications occurring after stroke and leading to a poor outcome. How this immune suppression is mediated is not still described but it has been postulated that inflammatory response may disable the immune system leading to immune suppression (87, 88). Alternatively, the brain in an attempt to prevent cerebral inflammation suppresses the immune system (89, 90). In both cases, the immunosuppressive state will increase the susceptibility of the patients to develop nosocomial infections, increasing the risk of poor long-term neurological state (Figure 5).

Pneumonia and urinary tract infections are the two most common infections occurring in patients with cerebrovascular accident, ranging around 23-65% and accounting for around 30% of mortality after stroke. Aspiration and dysphagia have been until present proposed as the main predictors of pneumonia, however and as just explained before, there is an increasing evidence that depression of the immune system is the main responsible of its development and that aspiration and dysphagia are only important contributors.

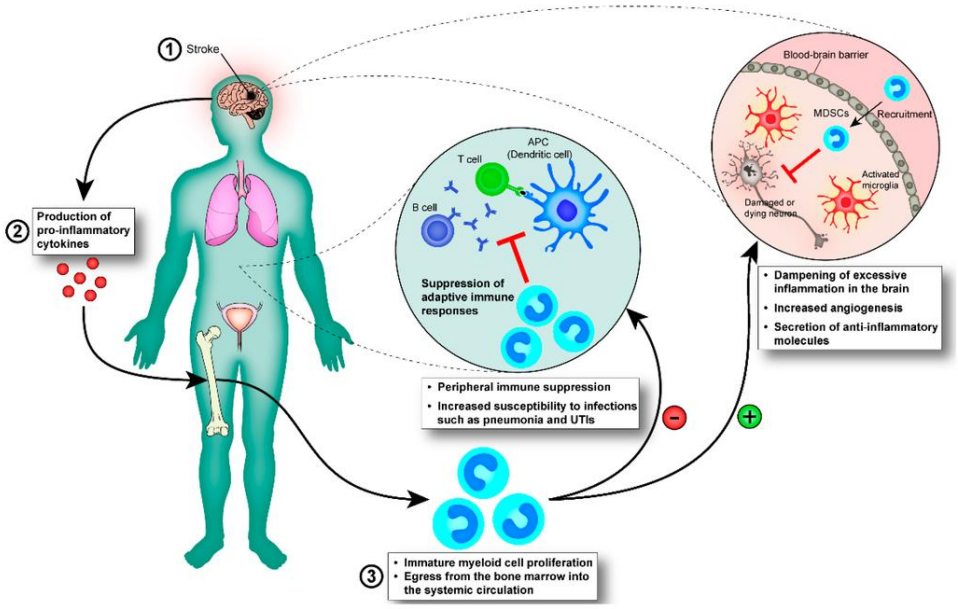

Figure 5: representation of mechanisms leading to post-stroke immunosupression.

The high rates of associated morbidity and mortality highlight the need of early initiation of antibiotic treatment once the infection is diagnosed. Several studies (PANTHERIS, PASS, and STROKE-INF) have evaluated the potential impact of prophylactic antibiotherapy in these patients, showing that it could be useful to reduce the number of nosocomial infections but without producing an impact in patient's outcome (91-93). Consequently, at present the only recommended guideline is to start with the treatment as soon as possible but only once the diagnosis is confirmed.

For this diagnosis step physicians face a challenging task as there are not well established criteria between the different studies. Signs of infection such as inflammation or fever can be present as a consequence of neurological damage in patients without infection. Furthermore, the use of aspirin can mask infection by decreasing fever. Chest radiographs are at present one of the most widely used methods to detect pneumonia, however they only present a sensitivity of 65% and they are only useful in advanced stages of infection in which the infiltrates have appeared (94, 95). Blood cultures, another very used clinical practice, are time consuming and the sensitivity is not high enough (96). Therefore, the discovery of biomarkers able to distinguish the patients at risk of infection development is crucial to apply new treatment strategies as soon as possible and to improve patient's outcome (97).

The relationship between inflammation and infection has been so widely supported that most of the proposed infection markers can be found in the list of molecules participating in the inflammatory cascade (76). A study including 113 patients tried to prospectively investigate the

different infection predefined biomarkers. It included laboratory markers, neuroimaging as well as daily screening clinical criteria. According to this investigation, body temperature, levels of white blood cells and IL-6 were significantly elevated one day after symptom onset in patients developing a post-stroke infection. Additionally, CRP levels were also significantly elevated three and five days after hospitalization in those patients presenting a serious infectious complication. The only significantly down-regulated molecule one day after admission, was the monozyte HLA-DR expression, which appeared to be lower in patients at risk of infection. Nevertheless, none of these markers was sensitive enough at the day of hospital admission to predict infection development (98).

In another retrospective study including 56 ischemic stroke patients and 5 transient ischemic attack patients (TIA), levels of lipopolysaccharide binding protein (LBP), interleukin-10 (IL-10), interleukin-6 (IL-6) and C-reactive protein (CRP) were evaluated in patients presenting or not post-stroke infection. Univariate analysis showed that between 12 hours and 7 days after the stroke onset, levels of LBP significantly differed between the two groups of patients. IL-10 concentrations only differed during the first day of hospitalization and IL-6 and CRP levels significantly differed at hospital admission but also one day, three days and seven days after. To evaluate if these markers were independently associated with infection development, the different parameters were adjusted for NIHSS, concluding that the only independent factors associated with infection were the IL-10, CRP and NIHSS (99).

Copeptin and procalcitonin are two well-known markers widely described and used as predictors of any type of infection. They have recently been described in stroke patients, showing that at hospital admission they were potential good predictors of pneumonia and urinary tract infections. Their accuracy in detecting patients at risk of infection development was quite similar to that shown in the same patients by WBC or CRP (97).

According to the results of the different studies, it is difficult to find a consensus between the capacity and accuracy of each different biomarker, which makes difficult the translation into the clinical practice. Some authors have consequently proposed that the combination of several markers with clinical scales could improve the individual prediction accuracy. Therefore, different score systems were developed to effectively evaluate the probability of presenting a post-stroke infection. While Kwon's score (100), Chumbler's 3-level score (101), Smith's ISAN score (102) are some of them, they have not been used in clinical practice due to the low sample size in which they have been performed as well as due to the lack of a validation step. A more recent combination of clinical parameters, the A2DS2 score performed on a high number

of stroke patients, showed that the combination of age, atrial fibrillation, dysphagia, sex and stroke severity could be a good tool to predict pneumonia after stroke (103, 104).

However, even the important efforts to find an ideal infection marker, with high values of SE (capacity to detect true positives) SP (capacity to detect true negatives), nothing is available. In the next section of the introduction we will explain the different steps followed during this thesis project to find an ideal biomarker able to predict infection in cerebrovascular accident patients.

3. Biomarker discovery workflow

Over the last decades, MS and proteomics, the field responsible of studying proteins, have advanced from qualitative to semiquantitative analysis. The improvement of the field has continued until now, being able at present to perform important quantitative analysis of entire proteome (105). Therefore, in this thesis project we have focused our attention on the discovery of biomarkers using proteomic approaches. For this purpose previously represented classical workflow of discovery verification, validation and clinical assessment was applied (Figure 6).

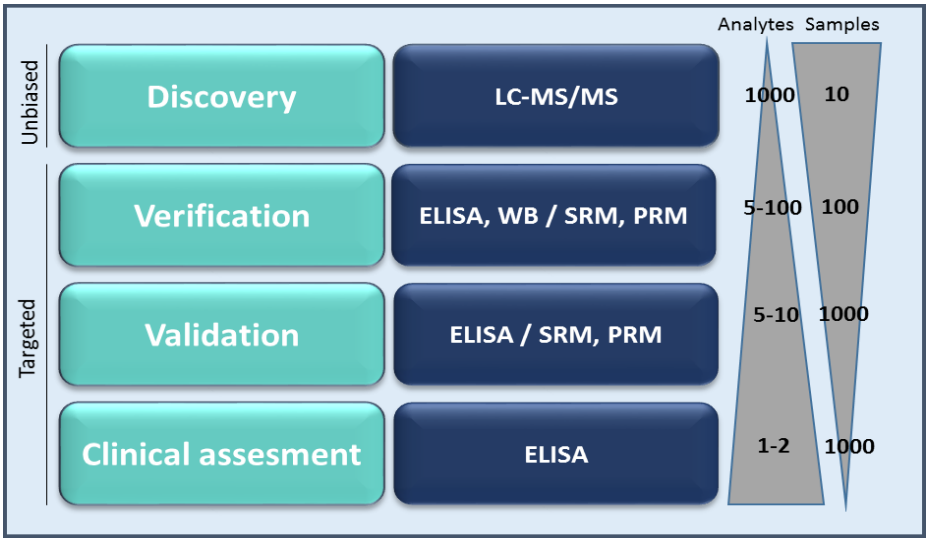

Figure 6: workflow representing the different biomarker discovery phases: discovery, verification, validation and clinical assessment

3.1. Proteomic biomarker discovery

For the discovery of new biomarkers different approaches can be used. Molecules related with disease pathogenesis or molecular mechanisms can be selected as potential candidates (hypothesis driven discovery).Untargeted MS approaches to produce a list of biomarkers have been also highly used in the last years (holistic driven discovery). In both cases, biomarker discovery steps will be performed using a sample cohort of less than 10 individuals, proposing more than 1000 candidates.

Quantitative proteomics, with in gel and non-gel based techniques, is a technique that allows to determine the quantity of a protein that there is in a specific sample. Among the in gel based techniques, two dimensional gel electrophoresis (2D-gel) was the most classical method to detect the difference protein expression between two or more clinical conditions (106). It combined the isoelectric focusing and the SDS-polyacrylamide gel electrophoresis so proteins are firstly separated according to their isoelectric point, and in the second dimension, according to their molecular weight. About 2000 spots can be simultaneously detected with the 2D-gel showing differences in their expression level, isoforms and/or post-translational modifications. However, the important drawbacks that are behind this technic have forced to develop different discovery alternatives. 2D-gels underestimate acid/basic or membrane proteins, it has low reproducibility and it is difficult to separe large (>200 kDa) and small (<10 kDa) proteins. Furthermore, and the most limiting cause for biomarker discovery is that only abundant proteins are identified, underestimating low abundant potential biomarkers (107).

Non-gel based techniques using MS have been proposed as promising alternatives to identify and quantify proteins. A mass spectrometer is composed by three different parts: the ion source, the mass analyzer and the detector. The ion source converts the analytes into gas-phase ions. The introduction of soft ionization techniques as ESI or MALDI, that allow the production of intact peptides or protein ions into the gas phase, has been the key point to increase the use of MS for proteomic analysis. ESI ionizes the molecules directly from a liquid solution (108), while in MALDI peptides or proteins are co-cristalized on a plate that is introduced in the MS (109). After the ionization process peptides arrive to the mass analyzer to be separated according to their m/z. Depending on the analytical performance or physical characteristics, different mass analyzers can be found. The most used ones in proteomics are: quadrupole (Q), ion trap (IT), time of flight (TOF), Fourier-transform ion cyclotron resonance (FTICR) and the orbitrap analyzer. Finally, m/z abundance of each ion will be measured in the detector.

To perform the discovery process different techniques can be applied in these kind of mass spectrometers: label-free and isotope labeled techniques are the two most known ones (110). Label free is a MS based method able to determine in a relative way the amount of proteins present in two or more samples. It does not modify the analytes and each sample is injected separately in the MS(111). Protein quantification can be performed considering that the intensity obtained in the MS is proportional to ion concentration or performing a spectral count of the identified proteins after tandem mass analysis. Stable isotope labeling is an approach in which the mass spectrometer is able to distinguish the same protein or peptide in different samples by using the increase in mass produced by mass tags (112). Proteins of different

samples are labeled and mixed separately to obtain a different isotopic profiles and are then analyzed using tandem mass spectrometry. By comparing the intensities of each identified peptide a relative quantification of each protein will be obtained.

To realize relative quantification however, isotope-coded affinity tags (ICAT)(113), isobaric labeling methods that include tandem mass tags (TMT)(114) and isobaric tags for relative and absolute quantification (iTRAQ)(115, 116), and stable isotope labeling with amino acids in cell culture (SILAC)(117) can be distinguished. In this thesis project we have mainly used the TMT approach that has been explained in detailed in Chapter 2.

Independently on the used technique to compare the samples, one of the main challenges of this biomarker discovery phase is to identify medium or low abundant proteins. Plasma is the main sample used to study stroke associated diseases. The accessibility and the low invasiveness of the collection technique made this fluid an excellent discovery sample. Nevertheless, the proteomic study of plasma samples among others, is a big challenge; due to the complexity and high dynamic range presented by the proteins. The identification of low-abundant proteins will be limited by the presence of highly abundant ones that represent the 99% of the total protein mass (118, 119). Therefore in most of the cases, before performing the comparison analysis, an antibody depletion pretreatment of the most abundant proteins of the sample could be necessary in order to adapt the sample to the discovery phase. In some cases the depletion step will remove some non-specifically-bound proteins being necessary to evaluate the benefit of this technique on each individual case.

3.2. **Potential biomarker verification**

Verification is an unavoidable step to evaluate the list of potential candidates obtained during the discovery phase (120). Each of those candidates is evaluated individually and their values of specificity, sensitivity and predictive values measured. Generally, verification step is performed in a larger cohort of around 50-100 of patients and using the same biological samples used in clinical application. Mass spectrometry based methods or immunoassays are the two preferable techniques to perform this step (121).

Immunoassays involve the already well known Western Blot, enzymed linked immunosorbent assay (ELISA) and the multiplex assays (122). During several years WB has been the method of choice for protein verification, however due to the high number of advantages, ELISA has then become the gold standard method: it is fast to perform, highly sensitive and highly specific(123). However, ELISA can only provide the measurement of a single analyte. Multiplex immunoassays

as MULTI-ARRAY (Meso Scale Discovery) or Bead-Based assays using the Bio-Plex system (Bio-Rad Laboratories) have been developed to overcome the limitation presented by ELISA and to allow the parallel measurement of several candidates. ELISA and multiplex immunoassays are easy to use and the required instrumentation is accessible. However, they also offer some limitations as for example cross-reactivities or protein-protein interactions than can modify or interfere with quantification results.

Targeted proteomics provides an alternative strategy. It has several advantages for the analysis of post-translational modifications, protein isoforms and genetic alterations. Furthermore, they can be also a good alternative when the needed antibodies required for the verification step are not available. The two most known targeted proteomic methods are: selected reaction monitoring and parallel reaction monitoring (Figure 8).

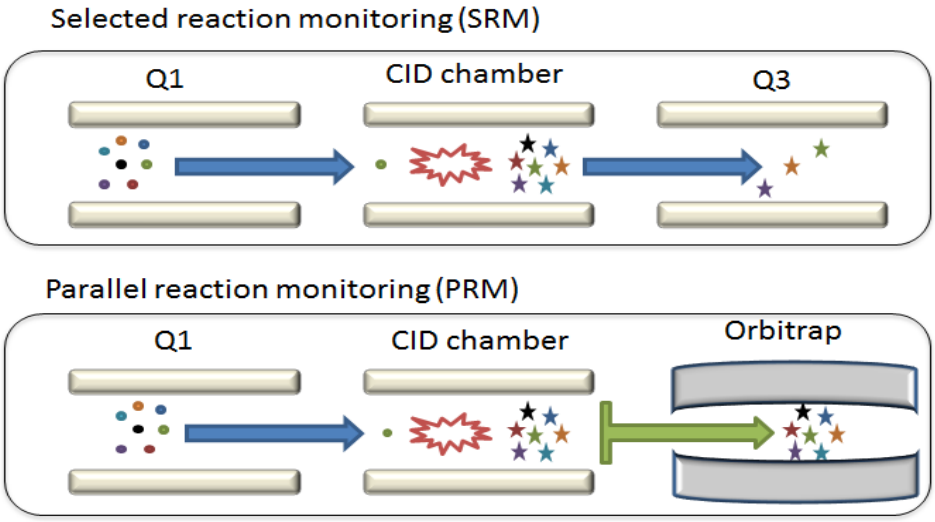

Figure 8: two different approaches in mass spectrometry-based targeted proteomics (a) selected reaction monitoring (SRM) where selected fragment ions from a single precursor are measured for the quantification, (b) parallel reaction monitoring (PRM) where a single precursor ion and entire fragment ions are selected.

Selected reaction monitoring (SRM) also known as multiple reaction monitoring (MRM) is able to quantify low abundant analytes and to target individual candidates. Analysis are usually performed on tripe quadrupole mass spectrometers, in which the first quadrupole is used to select the peptide of interest, the second quadrupole to fragment it and the third quadrupole to scan the produced fragments. Different transitions (precursor/fragment ion pairs) can be monitored with high selectivity during the time, however in some cases intensities coming from individual fragment product ions derived from a single precursor can be different. To obtain promising sensitive values, it is necessary to make a preselection of the most intense ions (Figure 8).

An efficient and faster alternative is parallel reaction monitoring (PRM), able to quantify different peptides with high values of sensitivity and specificity. PRM is usually performed in high-resolution hybrid quadrupole Orbitrap (Q-OT) or time of flight instruments. Precursor ions of interest are isolated in the quadrupole, fragmented in the cell of high energy collision dissociation (HCD) and analyzed using the Orbitrap (124, 125). The monitoring of product ions has a high resolution and consequently this method offers more specificity than SRM (Figure 8). Multiplexing up to 100 peptides is possible with both approaches.

3.3. **Biomarker validation**

The number of promising biomarkers arriving to the validation phase is low. The different approaches explained during the verification step could also be applied to perform the validation, being ELISA the method of choice in most of the cases. In this step, selected markers must be evaluated in thousands of patients and in multicentric studies to be sure that the full variation of the population is well considered (126). Sample size calculation is a critical first step of the validation process. Furthermore, in order to evaluate the performance of the validated biomarkers, statistical analysis should be performed. In most of the cases, biological data are not normally distributed and non-parametric tests are necessary to confirm that their efficiency is not produced by random variation. These kind of tests are mainly based on differences in medians: Mann-Whitney U test for example, equivalent to the parametric Student's t-test, compare the median of a continuous variable between two different conditions. When the two samples are related or matched, Wilcoxon signed-rank test is advised and when the number of conditions is higher than two, Kruskal-Wallis H test should be performed. To compare the efficacy of different diagnostic tests, receiver operating characteristic curves are an excellent possibility (Figure 9).

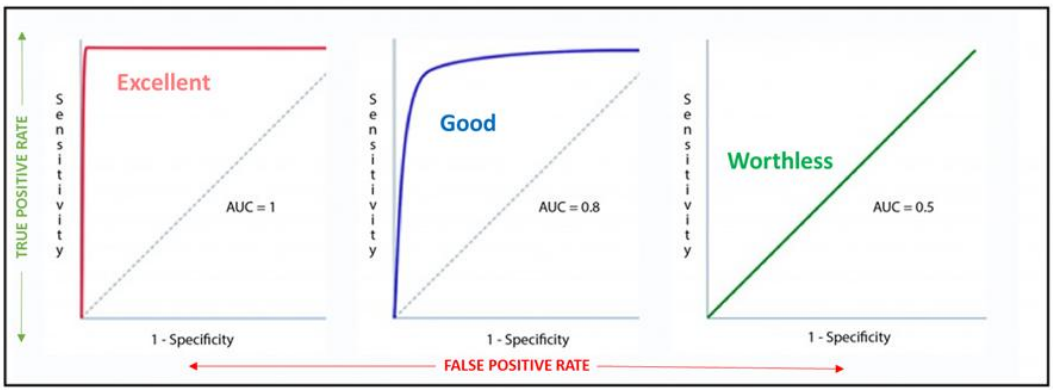

Figure 9: ROC curves representing excellent, good, and worthless tests plotted on the same graph.

Good biomarkers should present promising values of specificity (the percentage of correctly classified true negatives) and sensitivity (the percentage of correctly classified true positives).

The perfect combination would present 100% sensitivity (predict all people in the sick group as being sick) and 100% specificity (not predicting anyone from the healthy group as being sick). However, since this become almost impossible, a trade-off between both measures is usually calculated. In biomarker associated research it has become widely usual to report this trade-off sensitivity and specificity using receiver operating characteristics (ROC) curves, a representation created when plotting the true positive rate against the 1-specificity (127, 128). The area under the curve (AUC) of the ROC curve represents the total accuracy as well as the separation capacity of the selected biomarker, allowing to select optimal models and to cast away suboptimal ones.

The accuracy of the selected models can be importantly improved by combining individual biomarkers. Many different systems are available to do it: thresholds based methods, decision trees, regression methods, support vector machines or neural networks among others. In this thesis project however, we have used Panelomix tool, an approach that uses the iterative combination of biomarkers and thresholds (ICBT)(129). To accelerate the calculation when several biomarkers are combined, Panelomix selects different thresholds using the random forest method. Robustness of the panel and its performance are evaluated by cross-validation and ROC analysis.

3.4. **Biomarker clinical implementation**

The validation of biomarkers is usually an important and difficult step to reach, however the ultimate goal is its translation into the clinical practice (130).

Even if the performance of discovered and validated biomarkers is better than that obtained for the gold-standards of the disease, it does not automatically mean that the translation into the clinical practice is guaranteed. Implementation tests can develop during years. Furthermore, scientists are often not aware about the steps to follow. The cost-effectiveness of the biomarker it is not always easy to evaluate. Additionally, in some cases, patients may not wish to know about the outcome of the disease, especially if there is not an available treatment to improve it and physicians could resist to change their daily clinical practice. All these impediments have been tried to be reduced by the European Medical Research Council (EMRC), proposing a road map to follow. The initiative propose that biomarker research should follow a more standardized format, considering the needs as well as the perspectives of the different diseases to facilitate the use of biomarkers (131).

4. Aim of the project

The lack of a clinically accepted biomarker for infection prediction in aSAH and ischemic stroke patients represents a major limitation in the hospital management. At present, it is necessary to wait until bacterial culture result to have a final diagnosis of infection and in most of the cases it is too late to treat this complication without affecting their long-term poor outcome.

Considering the need of new alternatives, the aim of the present thesis project was to identify blood-markers able to accurately detect patients at risk of infection development. For this purpose we postulated that proteomic methods could be the best option to discover and validate early promising infection markers in aSAH and ischemic stroke patients.

Therefore, after introducing the disease and the state of the art of infection biomarkers in the first chapter of the thesis, in the second chapter, we prepared a chapter book, submitted to "*Neuromethods Series-Springer"* (2016) which describes the different applications of amine-reactive tandem mass tags in neuroproteomics, more concretely in neurodegenerative and cerebrovascular diseases.

Afterwards, we prepared an original article published in *Journal of Neurosurgery* (2015) presenting the capacity of neopterin to correlate with infection development in aSAH patients. The capacity to predict the outcome at one year was also evidenced. Nevertheless, one important drawback of these results was that neopterin was not early enough to predict infection development in aSAH patients and consequently, we decided to use proteomic methods to discover new earlier biomarkers.

In the chapter four, using the amino-reactive tandem mass tags, we were able to highlight the capacity of SAA to act as a promising predictor of infection in SAA at hospital admission. This results were published in *Journal of Proteome Research* (2015). Taking advantage of the capacity of SAA to predict infection such in an early way, we combined it with other clinical parameters (WBC, WFNS and age) postulating that the total accuracy could be improved.

As shown in the chapter V, an article published in *Journal of Translational Science* evidenced the capacity of a panel of biomarkers (SAA, WFNS, WBC, age) to improve the capacity of simple markers to predict infection in patients with aSAH.

Finally, we translated the promising results obtained during this thesis project to patients with ischemic stroke. Chapter VI is consequently, an original article published in *Clinical Proteomics* that shows the capacity of SAA to act as infection predictor marker at hospital admission in ischemic stroke patients.

The last chapter of this manuscript (chapter VII) gives a general discussion of the obtained results and proposes several perspectives for the future.

5. References

1. Feigin VL, Forouzanfar MH, Krishnamurthi R, Mensah GA, Connor M, Bennett DA, Moran AE, Sacco RL, Anderson L, Truelsen T, O'Donnell M, Venketasubramanian N, Barker-Collo S, Lawes CM, Wang W, Shinohara Y, Witt E, Ezzati M, Naghavi M, Murray C, (2014) Global and regional burden of stroke during 1990-2010: findings from the Global Burden of Disease Study 2010. Lancet 383: 245-254

2. Nagata K, Suzuki K, (2013) [Update on stroke epidemiology]. Brain Nerve 65: 857-870

3. Donnan GA, Fisher M, Macleod M, Davis SM, (2008) Stroke. The Lancet 371: 1612-1623

4. Li J, Wang D, Tao W, Dong W, Zhang J, Yang J, Liu M, (2016) Early consciousness disorder in acute ischemic stroke: incidence, risk factors and outcome. BMC neurology 16: 140

5. Howard VJ, (2013) Reasons Underlying Racial Differences in Stroke Incidence and Mortality. Stroke; a journal of cerebral circulation 44: S126-S128

6. Brønnum-Hansen H, Davidsen M, Thorvaldsen P, (2001) Long-Term Survival and Causes of Death After Stroke. Stroke; a journal of cerebral circulation 32: 2131-2136

7. Easton JD, Saver JL, Albers GW, Alberts MJ, Chaturvedi S, Feldmann E, Hatsukami TS, Higashida RT, Johnston SC, Kidwell CS, Lutsep HL, Miller E, Sacco RL, (2009) Definition and Evaluation of Transient Ischemic Attack. A Scientific Statement for Healthcare Professionals From the American Heart Association/American Stroke Association Stroke Council; Council on Cardiovascular Surgery and Anesthesia; Council on Cardiovascular Radiology and Intervention; Council on Cardiovascular Nursing; and the Interdisciplinary Council on Peripheral Vascular Disease: The American Academy of Neurology affirms the value of this statement as an educational tool for neurologists 40: 2276-2293

8. van der Worp HB, van Gijn J, (2007) Acute Ischemic Stroke. New England Journal of Medicine 357: 572-579

9. Thomson R, (2009) Evidence based implementation of complex interventions. BMJ 339

10. Whisnant JP, (1996) Effectiveness versus efficacy of treatment of hypertension for stroke prevention. Neurology 46: 301-307

11. Reynolds K, Lewis B, Nolen JL, Kinney GL, Sathya B, He J, (2003) Alcohol consumption and risk of stroke: A meta-analysis. JAMA 289: 579-588

12. Hankey GJ, (1999) Smoking and risk of stroke. J Cardiovasc Risk 6: 207-211

13. Group UKPDS, (1998) Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). The Lancet 352: 837-853

14. Kisialiou A, Grella R, Carrizzo A, Pelone G, Bartolo M, Zucchella C, Rozza F, Grillea G, Colonnese C, Formisano L, Lembo M, Puca AA, Vecchione C, (2014) Risk factors and acute ischemic stroke subtypes. Journal of the Neurological Sciences 339: 41-46

15. Adams HP, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, (1993) Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke 24

16. Lavallee P, Amarenco P, (2014) Stroke subtypes and interventional studies for transient ischemic attack. Front Neurol Neurosci 33: 135-146

17. Furie B, Furie BC, (2008) Mechanisms of Thrombus Formation. New England Journal of Medicine 359: 938-949

18. Singer OC, Humpich MC, Laufs H, Lanfermann H, Steinmetz H, Neumann-Haefelin T, (2006) Conjugate Eye Deviation in Acute Stroke. Incidence, Hemispheric Asymmetry, and Lesion Pattern 37: 2726-2732

19. Muir KW, Weir CJ, Murray GD, Povey C, Lees KR, (1996) Comparison of Neurological Scales and Scoring Systems for Acute Stroke Prognosis. Stroke; a journal of cerebral circulation 27: 1817-1820

20. Wilson JTL, Hareendran A, Grant M, Baird T, Schulz UGR, Muir KW, Bone I, (2002) Improving the Assessment of Outcomes in Stroke. Use of a Structured Interview to Assign Grades on the Modified Rankin Scale 33: 2243-2246

21. Jauch EC, Saver JL, Adams HP, Jr., Bruno A, Connors JJ, Demaerschalk BM, Khatri P, McMullan PW, Jr., Qureshi AI, Rosenfield K, Scott PA, Summers DR, Wang DZ, Wintermark M, Yonas H, (2013) Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke; a journal of cerebral circulation 44: 870-947

22. McLaughlin PD, Moloney F, O'Neill SB, James K, Crush L, Flanagan O, Maher MM, Wyse G, Fanning N, (2017) CT of the head for acute stroke: Diagnostic performance of a tablet computer prior to intravenous thrombolysis. J Med Imaging Radiat Oncol

23. Kidwell CS, Chalela JA, Saver JL, et al., (2004) Comparison of mri and ct for detection of acute intracerebral hemorrhage. JAMA 292: 1823-1830

24. Bederson JB, Connolly ES, Batjer HH, Dacey RG, Dion JE, Diringer MN, Duldner JE, Harbaugh RE, Patel AB, Rosenwasser RH, (2009) Guidelines for the Management of Aneurysmal Subarachnoid Hemorrhage: A Statement for Healthcare Professionals From a Special Writing Group of the Stroke Council, American Heart Association. Stroke; a journal of cerebral circulation 40: 994-1025

25. Southerland AM, (2017) Clinical Evaluation of the Patient With Acute Stroke. Continuum (Minneap Minn) 23: 40-61

26. Emberson J, Lees KR, Lyden P, Blackwell L, Albers G, Bluhmki E, Brott T, Cohen G, Davis S, Donnan G, Grotta J, Howard G, Kaste M, Koga M, von Kummer R, Lansberg M, Lindley RI, Murray G, Olivot JM, Parsons M, Tilley B, Toni D, Toyoda K, Wahlgren N, Wardlaw J, Whiteley W, del Zoppo GJ, Baigent C, Sandercock P, Hacke W, Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. The Lancet 384: 1929-1935

27. Roth JM, (2011) Recombinant tissue plasminogen activator for the treatment of acute ischemic stroke. Proceedings (Baylor University Medical Center) 24: 257-259

28. Scott Burgin W, Alexandrov AV, (2001) Deterioration following improvement with tPA therapy: Carotid thrombosis and reocclusion. Neurology 56: 568-570

29. Dubinsky R, Lai S-M, (2006) Mortality of stroke patients treated with thrombolysis: Analysis of nationwide inpatient sample. Neurology 66: 1742-1744

30. Alper BS, Malone-Moses M, McLellan JS, Prasad K, Manheimer E, (2015) Thrombolysis in acute ischaemic stroke: time for a rethink? BMJ : British Medical Journal 350

31. Caceres JA, Goldstein JN, (2012) Intracranial Hemorrhage. Emergency medicine clinics of North America 30: 771-794

32. Hemphill JC, Greenberg SM, Anderson CS, Becker K, Bendok BR, Cushman M, Fung GL, Goldstein JN, Macdonald RL, Mitchell PH, Scott PA, Selim MH, Woo D, (2015) Guidelines for the Management of Spontaneous Intracerebral Hemorrhage. A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association 46: 2032-2060

33. Feldmann E, Broderick JP, Kernan WN, Viscoli CM, Brass LM, Brott T, Morgenstern LB, Wilterdink JL, Horwitz RI, (2005) Major Risk Factors for Intracerebral Hemorrhage in the Young Are Modifiable. Stroke; a journal of cerebral circulation 36: 1881-1885

34. Feigin VL, Rinkel GJE, Lawes CMM, Algra A, Bennett DA, van Gijn J, Anderson CS, (2005) Risk Factors for Subarachnoid Hemorrhage. An Updated Systematic Review of Epidemiological Studies 36: 2773-2780

35. McDonald RJ, McDonald JS, Bida JP, Kallmes DF, Cloft HJ, (2012) Subarachnoid hemorrhage incidence in the United States does not vary with season or temperature. AJNR Am J Neuroradiol 33: 1663-1668

36. de Rooij NK, Linn FH, van der Plas JA, Algra A, Rinkel GJ, (2007) Incidence of subarachnoid haemorrhage: a systematic review with emphasis on region, age, gender and time trends. J Neurol Neurosurg Psychiatry 78: 1365-1372

37. Teunissen LL, Rinkel GJE, Algra A, van Gijn J, (1996) Risk Factors for Subarachnoid Hemorrhage. A Systematic Review 27: 544-549

38. van Gijn J, Kerr RS, Rinkel GJ, (2007) Subarachnoid haemorrhage. Lancet 369: 306-318

39. Molyneux AJ, Kerr RS, Yu LM, Clarke M, Sneade M, Yarnold JA, Sandercock P, (2005) International subarachnoid aneurysm trial (ISAT) of neurosurgical clipping versus endovascular coiling in 2143 patients with ruptured intracranial aneurysms: a randomised comparison of effects on survival, dependency, seizures, rebleeding, subgroups, and aneurysm occlusion. Lancet 366: 809-817

40. Inagawa T, (2001) Trends in Incidence and Case Fatality Rates of Aneurysmal Subarachnoid Hemorrhage in Izumo City, Japan, Between 1980–1989 and 1990–1998. Stroke; a journal of cerebral circulation 32: 1499-1507

41. Cahill J, Zhang JH, (2009) Subarachnoid Hemorrhage. Is It Time for a New Direction? 40: S86-S87

42. Linn FH, Rinkel GJ, Algra A, van Gijn J, (1998) Headache characteristics in subarachnoid haemorrhage and benign thunderclap headache. J Neurol Neurosurg Psychiatry 65: 791-793

43. Raper DMS, Starke RM, Komotar RJ, Allan R, Connolly ES, Jr., Seizures After Aneurysmal Subarachnoid Hemorrhage: A Systematic Review of Outcomes. World Neurosurgery 79: 682-690

44. De Marchis GM, Pugin D, Meyers E, Velasquez A, Suwatcharangkoon S, Park S, Falo MC, Agarwal S, Mayer S, Schmidt JM, Connolly ES, Claassen J, (2016) Seizure burden in subarachnoid hemorrhage associated with functional and cognitive outcome. Neurology 86: 253-260

45. Backes D, Rinkel GJE, Sturkenboom AJM, Vergouwen MDI, Time-dependent test characteristics of neck stiffness in patients suspected of nontraumatic subarachnoid haemorrhage. Journal of the Neurological Sciences 355: 186-188

46. Dubosh NM, Bellolio MF, Rabinstein AA, Edlow JA, (2016) Sensitivity of Early Brain Computed Tomography to Exclude Aneurysmal Subarachnoid Hemorrhage. A Systematic Review and Meta-Analysis

47. Carpenter CR, Hussain AM, Ward MJ, Zipfel GJ, Fowler S, Pines JM, Sivilotti ML, (2016) Spontaneous Subarachnoid Hemorrhage: A Systematic Review and Meta-analysis Describing the Diagnostic Accuracy of History, Physical Examination, Imaging, and Lumbar Puncture With an Exploration of Test Thresholds. Acad Emerg Med 23: 963-1003

48. Taylor RA, Singh Gill H, Marcolini EG, Meyers HP, Faust JS, Newman DH, (2016) Determination of a Testing Threshold for Lumbar Puncture in the Diagnosis of Subarachnoid Hemorrhage after a Negative Head Computed Tomography: A Decision Analysis. Acad Emerg Med 23: 1119-1127

49. van Gijn J, Rinkel GJE, (2001) Subarachnoid haemorrhage: diagnosis, causes and management. Brain 124: 249-278

50. Koh KM, Ng Z, Low SY, Chua HZ, Chou N, Low SW, Yeo TT, (2013) Management of ruptured intracranial aneurysms in the post-ISAT era: outcome of surgical clipping versus endovascular coiling in a Singapore tertiary institution. Singapore Med J 54: 332-338

51. Premananda RM, Ramesh N, Hillol KP, (2012) Functional outcome of microsurgical clipping compared to endovascular coiling. The Medical journal of Malaysia 67: 585-590

52. Citerio G, Gaini SM, Tomei G, Stocchetti N, (2007) Management of 350 aneurysmal subarachnoid hemorrhages in 22 Italian neurosurgical centers. Intensive Care Med 33: 1580-1586

53. Tholance Y, Barcelos GK, Perret-Liaudet A, Omar E, Carrillon R, Grousson S, Lieutaud T, Dailler F, Marinesco S, (2016) Placing intracerebral probes to optimise detection of delayed cerebral ischemia and allow for the prediction of patient outcome in aneurysmal subarachnoid haemorrhage. J Cereb Blood Flow Metab

54. Carr KR, Zuckerman SL, Mocco J Inflammation, Cerebral Vasospasm, and Evolving Theories of Delayed Cerebral Ischemia. Neurol Res Int. 2013;2013:506584. Epub 2013 Aug 22.,

55. Dhar R, Diringer MN, (2008) The burden of the systemic inflammatory response predicts vasospasm and outcome after subarachnoid hemorrhage. Neurocrit Care 8: 404-412

56. Provencio JJ, (2013) Inflammation in subarachnoid hemorrhage and delayed deterioration associated with vasospasm: a review. Acta Neurochir Suppl 115: 233-238

57. Yoshimoto Y, Tanaka Y, Hoya K, (2001) Acute systemic inflammatory response syndrome in subarachnoid hemorrhage. Stroke; a journal of cerebral circulation 32: 1989-1993

58. Frontera JA, Fernandez A, Schmidt JM, Claassen J, Wartenberg KE, Badjatia N, Connolly ES, Mayer SA, (2009) Defining vasospasm after subarachnoid hemorrhage: what is the most clinically relevant definition? Stroke; a journal of cerebral circulation 40: 1963-1968

59. Kimball MM, Velat GJ, Hoh BL, (2011) Critical care guidelines on the endovascular management of cerebral vasospasm. Neurocrit Care 15: 336-341

60. Furlan AJ, (2015) Endovascular Therapy for Stroke — It's about Time. New England Journal of Medicine 372: 2347-2349

61. Mehta Y, Gupta A, Todi S, Myatra SN, Samaddar DP, Patil V, Bhattacharya PK, Ramasubban S, (2014) Guidelines for prevention of hospital acquired infections. Indian Journal of Critical Care Medicine : Peer-reviewed, Official Publication of Indian Society of Critical Care Medicine 18: 149-163

62. Germanwala AV, Huang J, Tamargo RJ, Hydrocephalus After Aneurysmal Subarachnoid Hemorrhage. Neurosurgery Clinics 21: 263-270

63. Grasso G, Alafaci C, Macdonald RL, (2017) Management of aneurysmal subarachnoid hemorrhage: State of the art and future perspectives. Surg Neurol Int 8: 11

64. Aronson JK, (2005) Biomarkers and surrogate endpoints. British Journal of Clinical Pharmacology 59: 491-494

65. Strimbu K, Tavel JA, (2010) What are Biomarkers? Current opinion in HIV and AIDS 5: 463-466

66. Nalejska E, Mączyńska E, Lewandowska MA, (2014) Prognostic and Predictive Biomarkers: Tools in Personalized Oncology. Molecular Diagnosis & Therapy 18: 273-284

67. Frank R, Hargreaves R, (2003) Clinical biomarkers in drug discovery and development. Nat Rev Drug Discov 2: 566-580

68. Jickling GC, Sharp FR, (2011) Blood biomarkers of ischemic stroke. Neurotherapeutics 8: 349-360

69. Montaner J, (2006) Stroke biomarkers: Can they help us to guide stroke thrombolysis? Drug News Perspect 19: 523-532

70. Hirano K, Takashima S, Dougu N, Taguchi Y, Nukui T, Konishi H, Toyoda S, Kitajima I, Tanaka K, (2012) Study of hemostatic biomarkers in acute ischemic stroke by clinical subtype. J Stroke Cerebrovasc Dis 21: 404-410

71. Kaura V, Bonner S, (2012) Subarachnoid haemorrhage: Early clinical indicators and biomarkers. Trends in Anaesthesia and Critical Care 2: 42-47

72. Elkind MSV, (2008) Inflammatory markers and stroke. Current Cardiology Reports 11: 12

73. Elkind MS, Luna JM, Coffey CS, McClure LA, Liu KM, Spitalnik S, Paik MC, Roldan A, White C, Hart R, Benavente O, (2010) The Levels of Inflammatory Markers in the Treatment of Stroke study (LIMITS): inflammatory biomarkers as risk predictors after lacunar stroke. International journal of stroke : official journal of the International Stroke Society 5: 117-125

74. del Zoppo GJ, Becker KJ, Hallenbeck JM, (2001) Inflammation after stroke: is it harmful? Arch Neurol 58: 669-672

75. Carr KR, Zuckerman SL, Mocco J, (2013) Inflammation, cerebral vasospasm, and evolving theories of delayed cerebral ischemia. Neurol Res Int 2013: 506584

76. Antonino T, Domenico Di R, Riccardo di S, Antonio P, Giuseppe L, (2008) Inflammatory Cytokines in Acute Ischemic Stroke. Current Pharmaceutical Design 14: 3574-3589

77. Tyrrell HCAEaPJ, (2002) Inflammation and infection in clinical stroke. Journal of Cerebral Blood Flow & Metabolism 22: 1399–1419

78. Dziedzic T, Slowik A, Szczudlik A, (2003) Interleukin-6 and Stroke: Cerebral Ischemia Versus Nonspecific Factors Influencing Interleukin-6. Stroke; a journal of cerebral circulation 34: e229-e230

79. Katsnelson M, Rundek T, (2011) Chemokines and stroke: the subcellular harbingers of apoplexy? Neurology 77: 1116-1117

80. Rosell A, Cuadrado E, Ortega-Aznar A, Hernández-Guillamon M, Lo EH, Montaner J, (2008) MMP-9–Positive Neutrophil Infiltration Is Associated to Blood–Brain Barrier Breakdown and Basal Lamina Type IV Collagen Degradation During Hemorrhagic Transformation After Human Ischemic Stroke. Stroke; a journal of cerebral circulation 39: 1121-1126

81. Vukasovic I, Tesija-Kuna A, Topic E, Supanc V, Demarin V, Petrovcic M, (2006) Matrix metalloproteinases and their inhibitors in different acute stroke subtypes. Clin Chem Lab Med 44: 428-434

82. Simundic AM, Basic V, Topic E, Demarin V, Vrkic N, Kunovic B, Stefanovic M, Begonja A, (2004) Soluble adhesion molecules in acute ischemic stroke. Clin Invest Med 27: 86-92

83. Lad SP, Hegen H, Gupta G, Deisenhammer F, Steinberg GK, (2012) Proteomic biomarker discovery in cerebrospinal fluid for cerebral vasospasm following subarachnoid hemorrhage. J Stroke Cerebrovasc Dis 21: 30-41

84. Parkinson D, Stephensen S, (1984) Leukocytosis and subarachnoid hemorrhage. Surg Neurol 21: 132-134

85. Benakis C, Garcia-Bonilla L, Iadecola C, Anrather J, (2014) The role of microglia and myeloid immune cells in acute cerebral ischemia. Front Cell Neurosci 8

86. Romero FR, Bertolini Ede F, Figueiredo EG, Teixeira MJ, (2012) Serum C-reactive protein levels predict neurological outcome after aneurysmal subarachnoid hemorrhage. Arq Neuropsiquiatr 70: 202-205

87. Brea D, Sobrino T, Blanco M, Fraga M, Agulla J, Rodriguez-Yanez M, Rodriguez-Gonzalez R, Perez de la Ossa N, Leira R, Forteza J, Davalos A, Castillo J, (2009) Usefulness of haptoglobin and serum amyloid A proteins as biomarkers for atherothrombotic ischemic stroke diagnosis confirmation. Atherosclerosis 205: 561-567

88. Emsley HC, Smith CJ, Gavin CM, Georgiou RF, Vail A, Barberan EM, (2003) An early and sustained peripheral inflammatory response in acute ischaemic stroke: relationships with infection and atherosclerosis. J Neuroimmunol 139

89. Offner H, Subramanian S, Parker SM, Afentoulis ME, Vandenbark AA, Hurn PD, (2006) Experimental stroke induces massive, rapid activation of the peripheral immune system. J Cereb Blood Flow Metab 26: 654-665

90. Emsley HC, Hopkins SJ, (2010) Post-stroke immunodepression and infection: an emerging concept. Infect Disord Drug Targets 10: 91-97

91. Chamorro A, Urra X, Planas AM, (2007) Infection after acute ischemic stroke: a manifestation of brain-induced immunodepression. Stroke 38

92. Harms H, Grittner U, Droge H, Meisel A, (2013) Predicting post-stroke pneumonia: the PANTHERIS score. Acta Neurol Scand 128: 178-184

93. Kalra L, Irshad S, Hodsoll J, Simpson M, Gulliford M, Smithard D, Patel A, Rebollo-Mesa I, (2015) Prophylactic antibiotics after acute stroke for reducing pneumonia in patients with dysphagia (STROKE-INF): a prospective, cluster-randomised, open-label, masked endpoint, controlled clinical trial. The Lancet 386: 1835-1844

94. Westendorp WF, Vermeij JD, Zock E, Hooijenga IJ, Kruyt ND, Bosboom HJ, Kwa VI, Weisfelt M, Remmers MJ, ten Houten R, Schreuder AH, Vermeer SE, van Dijk EJ, Dippel DW, Dijkgraaf MG, Spanjaard L, Vermeulen M, van der Poll T, Prins JM, Vermeij FH, Roos YB, Kleyweg RP, Kerkhoff H, Brouwer MC, Zwinderman AH, van de Beek D, Nederkoorn PJ, (2015) The Preventive Antibiotics in Stroke Study (PASS): a pragmatic randomised open-label masked endpoint clinical trial. Lancet 385: 1519-1526

95. Burillo A, Bouza E, (2014) Use of rapid diagnostic techniques in ICU patients with infections. BMC Infectious Diseases 14: 593

96. Albrich WC, Harbarth S, (2015) Pros and cons of using biomarkers versus clinical decisions in start and stop decisions for antibiotics in the critical care setting. Intensive Care Med 41: 1739-1751

97. Gumbinger C, Hug A, Murle B, Berger B, Zorn M, Becker KP, Zimmermann S, Dalpke AH, Veltkamp R, (2013) Early blood-based microbiological testing is ineffective in severe stroke patients. J Neurol Sci 325: 46-50

98. Fluri F, Morgenthaler NG, Mueller B, Christ-Crain M, Katan M, (2012) Copeptin, Procalcitonin and Routine Inflammatory Markers–Predictors of Infection after Stroke. PloS one 7: e48309

99. Wartenberg KE, Stoll A, Funk A, Meyer A, Schmidt JM, Berrouschot J, (2011) Infection after Acute Ischemic Stroke: Risk Factors, Biomarkers, and Outcome. Stroke research and treatment 2011

100. Worthmann H, Tryc AB, Dirks M, Schuppner R, Brand K, Klawonn F, Lichtinghagen R, Weissenborn K, (2015) Lipopolysaccharide binding protein, interleukin-10, interleukin-6 and C-reactive protein blood levels in acute ischemic stroke patients with post-stroke infection. Journal of Neuroinflammation 12: 13

101. Kwon H-M, Jeong S-W, Lee S-H, Yoon B-W, The pneumonia score: A simple grading scale for prediction of pneumonia after acute stroke. American Journal of Infection Control 34: 64-68

102. Chumbler NR, Williams LS, Wells CK, Lo AC, Nadeau S, Peixoto AJ, Gorman M, Boice JL, Concato J, Bravata DM, (2010) Derivation and Validation of a Clinical System for Predicting Pneumonia in Acute Stroke. Neuroepidemiology 34: 193-199

103. Smith CJ, Bray BD, Hoffman A, Meisel A, Heuschmann PU, Wolfe CD, Tyrrell PJ, Rudd AG, (2015) Can a novel clinical risk score improve pneumonia prediction in acute stroke care? A UK multicenter cohort study. J Am Heart Assoc 4: e001307

104. Sellars C, Bowie L, Bagg J, Sweeney MP, Miller H, Tilston J, (2007) Risk factors for chest infection in acute stroke: a prospective cohort study. Stroke 38

105. Hoffmann S, Malzahn U, Harms H, Koennecke HC, Berger K, Kalic M, Walter G, Meisel A, Heuschmann PU, (2012) Development of a clinical score (A2DS2) to predict pneumonia in acute ischemic stroke. Stroke; a journal of cerebral circulation 43: 2617-2623

106. Li Y, Song B, Fang H, Gao Y, Zhao L, Xu Y, (2014) External validation of the A2DS2 score to predict stroke-associated pneumonia in a Chinese population: a prospective cohort study. PLoS One 9: e109665

107. Craft GE, Chen A, Nairn AC, (2013) Recent advances in quantitative neuroproteomics. Methods 61: 186-218

108. Rabilloud T, Lelong C, (2011) Two-dimensional gel electrophoresis in proteomics: A tutorial. Journal of Proteomics 74: 1829-1841

109. Bunai K, Yamane K, (2005) Effectiveness and limitation of two-dimensional gel electrophoresis in bacterial membrane protein proteomics and perspectives. Journal of Chromatography B 815: 227-236

110. Fenn J, Mann M, Meng C, Wong S, Whitehouse C, (1989) Electrospray ionization for mass spectrometry of large biomolecules. Science 246: 64-71

111. Karas M, Hillenkamp F, (1988) Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Analytical chemistry 60: 2299-2301

112. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B, (2007) Quantitative mass spectrometry in proteomics: a critical review. Analytical and Bioanalytical Chemistry 389: 1017-1031

113. Asara J, Christofk H, Freimark L, Cantley L, (2008) A label-free quantification method by MS/MS TIC compared to SILAC and spectral counting in a proteomics screen. Proteomics 8: 994-999

114. Morton TH (2010) Isotopic Labelling in Mass Spectrometry* A2 - Lindon, John CEncyclopedia of Spectroscopy and Spectrometry (Second Edition). Academic Press, Oxford, pp. 1237-1246

115. Xiao Z, Veenstra TD, (2008) Comparison of protein expression by isotope-coded affinity tag labeling. Methods Mol Biol 428: 181-192

116. Venable JD, Dong M-Q, Wohlschlegel J, Dillin A, Yates JR, (2004) Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra. Nat Meth 1: 39-45

117. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ, (2004) Multiplexed Protein Quantitation in Saccharomyces cerevisiae Using Amine-reactive Isobaric Tagging Reagents. Molecular & Cellular Proteomics 3: 1154-1169

118. Evans C, Noirel J, Ow SY, Salim M, Pereira-Medrano AG, Couto N, Pandhal J, Smith D, Pham TK, Karunakaran E, Zou X, Biggs CA, Wright PC, (2012) An insight into iTRAQ: where do we stand now? Anal Bioanal Chem 404: 1011-1027

119. Kani K, (2017) Quantitative Proteomics Using SILAC. Methods Mol Biol 1550: 171-184

120. Mitchell P, (2010) Proteomics retrenches. Nat Biotech 28: 665-670

121. Hortin GL, Sviridov D, (2010) The dynamic range problem in the analysis of the plasma proteome. Journal of Proteomics 73: 629-636

122. Hunter DJ, Losina E, Guermazi A, Burstein D, Lassere MN, Kraus V, (2010) A pathway and approach to biomarker validation and qualification for osteoarthritis clinical trials. Curr Drug Targets 11: 536-545

123. Whiteaker JR, Lin C, Kennedy J, Hou L, Trute M, Sokal I, Yan P, Schoenherr RM, Zhao L, Voytovich UJ, Kelly-Spratt KS, Krasnoselsky A, Gafken PR, Hogan JM, Jones LA, Wang P, Amon L, Chodosh LA, Nelson PS, McIntosh MW, Kemp CJ, Paulovich AG, (2011) A targeted proteomics-based pipeline for verification of biomarkers in plasma. Nat Biotech 29: 625-634

124. Cummings J, Raynaud F, Jones L, Sugar R, Dive C, (2010) Fit-for-purpose biomarker method validation for application in clinical trials of anticancer drugs. Br J Cancer 103: 1313-1317

125. Haverland N, Pottiez G, Wiederin J, Ciborowski P, (2010) Immunoreactivity of anti-gelsolin antibodies: implications for biomarker validation. Journal of Translational Medicine 8: 137

126. Bourmaud A, Gallien S, Domon B, (2016) Parallel reaction monitoring using quadrupole-Orbitrap mass spectrometer: Principle and applications. Proteomics 16: 2146-2159

127. Rauniyar N, (2015) Parallel Reaction Monitoring: A Targeted Experiment Performed Using High Resolution and High Mass Accuracy Mass Spectrometry. Int J Mol Sci 16: 28566-28581

128. Cummings J, Ward TH, Greystoke A, Ranson M, Dive C, (2008) Biomarker method validation in anticancer drug development. Br J Pharmacol 153: 646-656

129. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, Muller M, (2011) pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 12: 1471-2105

130. Hajian-Tilaki K, (2013) Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation. Caspian J Intern Med 4: 627-635

131. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, Müller M, (2013) PanelomiX: A threshold-based algorithm to create panels of biomarkers. Translational Proteomics 1: 57-64

132. Varghese S, Lao–Sirieix P, Fitzgerald RC, (2012) Identification and Clinical Implementation of Biomarkers for Barrett's Esophagus. Gastroenterology 142: 435-441.e432

133. Mischak H, Ioannidis JP, Argiles A, Attwood TK, Bongcam-Rudloff E, Broenstrup M, Charonis A, Chrousos GP, Delles C, Dominiczak A, Dylag T, Ehrich J, Egido J, Findeisen P, Jankowski J, Johnson RW, Julien BA, Lankisch T, Leung HY, Maahs D, Magni F, Manns MP, Manolis E, Mayer G, Navis G, Novak J, Ortiz A, Persson F, Peter K, Riese HH, Rossing P, Sattar N, Spasovski G, Thongboonkerd V, Vanholder R, Schanstra JP, Vlahou A, (2012) Implementation of proteomic biomarkers: making it work. Eur J Clin Invest 42: 1027-1036

Chapter II

Applications of amine-reactive tandem mass tags (TMT) in human neuroproteomics

Applications of amine-reactive tandem mass tags (TMT) in human neuroproteomics

Linnéa Lagerstedt, Leire Azurmendi, Jean-Charles Sanchez

The first chapter of this manuscript is focused on the impact that the amine-reactive tandem mass tags (TMT) have in neuroproteomics. During this thesis project, the TMT has been the method of choice to perform all the proteomic discovery experiments I have performed and consequently, in this chapter book we described the method in detail.

Afterwards, applications in general neurodegenerative disorders are explained to end with the applications in cerebrovascular diseases. All the material and methods needed to perform a proteomic experiment are detailed rigorously.

The chapter book will be soon published in Neuromethods Series-Springer. Linnea Lagerstedt and me contributed equally to this work writing together the chapter.

Applications of amine-reactive tandem mass tags (TMT) in human neuroproteomics

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Running title

TMT 10-plex in human neuroproteomics

Abstract

Neuroproteomics is a complex field of life sciences due to the high complexity of the brain. This area comprises different pathophysiological conditions such as normal neurodevelopment, neurovascular disorders and neurodegenerative disorders. A massive amount of studies have been performed using proteomics to increase the knowledge in this topic. However, there are still a lot more to explore. The most common proteomic techniques for investigating the different stages and conditions in neurodevelopment and diseases have mainly been based on two-dimensional gel electrophoresis (2-DE). More recently, the use of amine reactive tandem mass tags (TMT) has also contributed to increase the understanding of the brain and associated disorders. The TMT can simultaneously compare up to 10 samples and is compatible with a variety of biological samples. The proteins are labelled, pooled and co-eluted and analysed by LC-MS/MS. The multiplexing allows different designs and comparisons between the samples. Therefore the method is highly recommendable for e.g. biomarker discovery in the neuroproteomic field. In this chapter the TMT 10-plex method will be detailed for use with three different brain proximal samples; cerebrospinal fluid (CSF), brain tissue and neurons.

1. Introduction

Neuroproteomics is a branch of proteomics in charge of studying normal neurodevelopment, neurovascular- and neurodegenerative disorders as well as the central and peripheral nervous system (CNS) structure, functions and biological mechanisms (132, 133). The brain complexity and heterogeneity present unique challenges for neuroproteomics. Firstly, brain proteins can undergo a variety of post-translational modifications such as phosphorylation, ubiquitinylation, glycosylation or oxidation producing different regulations in the distinct brain regions. In addition to this, there is a high brain cellular heterogeneity; different regions are composed by different cellular types. Finally, the different modules of the brain are defined by their cognitive function, highlighting dissimilar tasks depending on the brain region; hippocampus for example, has an important role in consolidation of memory; the amygdala is mostly responsible of coordinating emotional behaviours; the striatum is in control of decision making, planning and perception and finally cerebral cortex has a key role in language, attention and consciousness (105). All these features generate a number of challenges to investigate the brain at the molecular level. Until present, the brain has been the most complex and unknown organ of our body. At the proteomics strategy level, the transition from two-dimensional gel electrophoresis (2-DE) to MS-based neuroproteomic studies using amine reactive tandem mass tags (ITRAQ - TMT) has led to new insights into the brain comprehension (134). In this chapter, we will focus our attention on studies that used TMT to identify CNS disease biomarkers and disease biological mechanisms.

1.1. TMT labelling method

The TMT labelling technique permits several samples to be analysed simultaneously by mass spectrometry. The number of samples that can be labelled by different tags in a single experiment spends from 2- to 10-plex. The tags used in TMT are composed of three distinct parts; a reporter group, a balance group, and a reactive group. The reactive group binds to free amino-terminus peptides and epsilon-amino functions of lysine residues (135). Each reporter group has a specific molecular mass permitting the differentiation of the tags after fragmentation however the addition of a balance group equal the total mass to be the same for all tags (135). For the 6-plex, five heavy isotopes (13C or 15N) are used to generate 6 different isobaric tags (135). To obtain 10 tags, four isotope variants of the 6-plex tags were developed (figure 1) (135). During the experiment, the samples to be compared will be tagged separately and thereafter pooled together for simultaneous identification and quantification (136). Due to the similar molecular composition, the TMT tags have the same physiochemical properties such

as isoelectric point (p*I*), hydrophobicity and total mass (136). Due to the common properties, the differentially labelled peptides will co-elute and appear as one peak in the MS1 spectra which lead to a more accurate quantification (135, 137). Tags will be cleaved using HCD fragmentation for quantification whereas identification is performed using HCD, CID or ETD (135, 137-141). After fragmentation, each reporter group appears as an independent peak in MS/MS spectra due to the unique molecular mass enabling relative quantification of the corresponding sample (figure 2) (135, 137, 142).

The application field of the TMT method is tremendous due to its capacity to simultaneously quantify and thereby compare up to 10 different samples. Furthermore, the TMT can be used on a multitude of different samples such as CSF, blood, cells and tissues. Therefore the method can be applied on a large number of areas including biomarker discovery studies in neuroproteomics. The multiplexing allows several different types of design to be realized such as case control studies or longitudinal studies among others. The TMT has previously been studied in several different cerebral pathologies described here below.

1.2. **Applications of TMT in neurodegenerative diseases**

Protein changes were studied using TMT in two of the most known neurodegenerative disorders: Alzheimer disease (AD) and Parkinson disease (PD).

In the case of Alzheimer disease, it is widely known that the disease process is associated with an accumulation of β-amyloid peptides. Nevertheless, the etiology is still poorly understood (143). Therefore, in the last decades several proteomic studies were performed to elucidate the associated mechanisms as well as the biomarkers that could improve the management of these patients. In cerebral cortices of mice with Aβ deposition, TMT was used to reveal the molecular mechanisms underlying Aβ clearance (144). The proteome of APP/PS1 double transgenic mice and wild type mice was compared obtaining a novel profile of altered proteins in AD model (144). Furthermore, in plasma samples of AD patients, the amino-reactive tandem mass tag was used to discover biomarkers able to discriminate between AD patients and control patients (145).

Data on Parkinson disease, the second most common neurodegenerative disorder, has also importantly been produced using mass spectrometry labelling methods. Anatomically, the main characteristic of PD is the loss of dopaminergic neurons in substantia nigra, which is usually accompanied by motor symptoms. To better understand the pathogenic mechanisms of the disease, the proteome of substantia nigra was compared in PD and control patients using the

TMT-6plex. With this approach authors were able to suggest a role for nebulette overexpression in neurodegeneration produced by PD (146).

The TMT methodology has not only been used in neurodegenerative disorders. Other neurovascular disorders such as stroke and subarachnoid haemorrhage have also benefit from this methodology.

1.3. **Applications of TMT in cerebrovascular diseases**

The high rate of mortality and morbidity associated to cerebrovascular diseases, such as stroke or subarachnoid haemorrhage, has increased the number of proteomic studies in an attempt to identify new associated biomarkers.

Stroke is the leading cause of disability and the second cause of death world-wide. The early detection of patient's diagnosis could help in decision making processes leading to a better management and to an improvement in the associated prognosis. TMT 2-plex and extracellular brain fluid of stroke patients were used to compare the infarct core with the unaffected contralateral region of the brain. This allowed to identify specific biomarkers of the affected region, highlighting the neuroprotective and apoptotic mechanisms that could be induced after a brain ischemic event (147).

In aneurysmal subarachnoid haemorrhage, a subtype of stroke, the high rates of mortality and morbidity are usually due to the initial haemorrhage. However, complications such as infection or delayed cerebral ischemia (DCI) developed during hospitalization are responsible of a high number of deaths (148). A 6-plex TMT analysis has compared the plasma proteome of patients developing infectious complications from those without in order to detect as early as possible patient at risk of being infected. Several biomarkers such as serum amyloid A were able to distinguish these patients, improving patient management and consequently in patient outcome (149).

In the next sections, the TMT 10-plex method will be detailed to illustrate each step of the workflow. It is separated in 8 main parts; "sample preparation", "Bradford assay", "reduction, alkylation and protein digestion", "TMT labelling", "purification", "fractionation", "LC-MS/MS" and "data analysis".

2. Material

The following material, or equivalent, is necessary to perform a TMT analysis. The material is described in the different main parts as used during the analysis.

2.1. Sample preparation

Neuroproteomic using TMT analysis has been used in a large number of different human samples, most of them being taken post-mortem. In this chapter we will focus our attention on the preparation of three CNS derived samples: cerebrospinal fluid (CSF), cerebral tissue and neurons.

2.1.1. CSF (150)

- Millex-HV filters, Merck Millipore (Schaffhausen, Switzerland)
- HPLC Alliance HT, Waters (Baden-Dättwil, Switzerland)
- MARS column, Agilent Technologies (Santa Clara, CA, USA)
- MARS buffer A and B, Agilent Technologies (Santa Clara, CA, USA)
- SpeedVac concentrator, Savant SPD111V, Thermo Scientific (Waltham, MA, USA)

2.1.2. Cerebral tissue (146)

- Microtome, Leica (Muttenz, Switzerland)
- Triethylammonium hydrogen carbonate buffer (TEAB) 1M, Sigma-Aldrich (Milwaukee, WI, USA)
- Sodium dodecyl sulfate (SDS ≥98%), Sigma-Aldrich (Milwaukee, WI, USA)
- CHAPS, Sigma-Aldrich (Milwaukee, WI, USA)
- Protease Inhibitor Cocktail Tablets, Roche (Basel, Switzerland)
- Sonicator, Thermo Scientific (Waltham, MA, USA)

Solubilisation buffer: TEAB 100mM (pH 8), SDS 20% (w/v), CHAPS 4% (w/v), Proteases Inhibitor 1x

2.1.3. Neurons (151)

- Sequencing Grade Modified Trypsin, Promega (Madison, WI, USA)
- HBSS, no calcium, no magnesium, Thermo Scientific (Waltham, MA, USA)
- HBSS, with calcium and magnesium, Thermo Scientific (Waltham, MA, USA)
- Protease Inhibitor Cocktail Tablets, Roche (Basel, Switzerland)
- Neurobasal medium plus B27 supplement, Invitrogen (Carlsbad, CA)

- Tris(hydroxymethyl)aminomethane hydrochloride, Sigma-Aldrich (Milwaukee, WI, USA)
- Hydrochloric acid (HCl 25%), Merck Millipore (Schaffhausen, Switzerland)
- Triton X-100, Sigma-Aldrich (Milwaukee, WI, USA)
- Sodium fluoride (NaF), Sigma-Aldrich (Milwaukee, WI, USA)
- Sodium orthovanadate (Na3VO4), Sigma-Aldrich (Milwaukee, WI, USA)
- Sonicator, Thermo Scientific (Waltham, MA, USA)
- Chloroform, Sigma-Aldrich (Milwaukee, WI, USA)
- Methanol, Sigma-Aldrich (Milwaukee, WI, USA)
- RapiGest SF Surfactant, Waters (Baden-Dättwil, Switzerland)

Lysis buffer: Tris-HCl 50mM (pH 8.5), Triton X-100 2% (w/v), NaF 10mM, Na_3VO_4 1mM

2.2. **Bradford assay**

- Bovine Serum Albumin (BSA), Sigma-Aldrich (Milwaukee, WI, USA)
- Dye Reagent Concentrate, Bio-Rad (California, USA)
- Cuvettes with 1 cm path length matched to laboratory spectrophotometer, Bio-Rad (California, USA)
- Spectrophotometer set to 595nm, Ultrospec 2100 Pro, Amersham Biosciences (Buckinghamshire, United Kingdom)

2.3. **Reduction, alkylation and protein digestion**

- Triethylammonium hydrogen carbonate buffer (TEAB) 1M, Sigma-Aldrich (Milwaukee, WI, USA)
- Hydrochloric acid (HCl 25%), Merck Millipore (Schaffhausen, Switzerland)
- 744 pH Meter, Metrohm (Herisau, Switzerland)
- Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), Sigma-Aldrich (Milwaukee, WI, USA)
- Iodoacetamide (IAA), Sigma-Aldrich (Milwaukee, WI, USA)

Sequencing Grade Modified Trypsin, Promega (Madison, WI, USA)

2.4. **TMT labelling**

- TMT 10-plex™ reagents, Thermo Scientific (Waltham, MA, USA)
- Acetonitrile (ACN), Chromasolv® for HPLC (≥99.9%), Sigma-Aldrich (Milwaukee, WI, USA)
- Hydroxylamine solution 50 wt% in water, Sigma-Aldrich (Milwaukee, WI, USA)

2.5. **Purification (Macro and Micro SpinColumn)**

- C18 Macro SpinColumn, Harvard Apparatus (Holliston, MA, USA)
- C18 Micro SpinColumn, Harvard Apparatus (Holliston, MA, USA)
- Acetonitrile (ACN), Chromasolv® for HPLC (≥99.9%), Sigma-Aldrich (Milwaukee, WI, USA)
- Water, Romil-SPS Super purity solvent (≥99.999%), Romil (Cambridge, England)
- Formic acid (FA), ULC/MS grade (99%), Biosolve (Valkenswaard, Netherlands) to be collected with a glass syringe, Hamilton (Bondaduz, Switzerland).
- pH-indicator paper pH 1-10 Universal indicator, Merck Millipore (Schaffhausen, Switzerland)
- SpeedVac concentrator, Savant SPD111V, Thermo Scientific (Waltham, MA, USA)

Solutions: 50% H_2O+50% ACN+0.1% FA, 95% H_2O+5% ACN+0.1% FA, 10% FA

2.6. **Fractionation**

- A 3100 OFFGEL Fractionator, Agilent Technologies (Santa Clara, CA, USA) with a 12 well fractionator.
- IPG strip, Immobiline DryStrip pH 3-10, 13cm, GE Healthcare Bio-Sciences AB (Uppsala, Sweden)
- Glycerol 87%, Merck Millipore (Schaffhausen, Switzerland)
- IPG buffer, pH 3-10, GE Healthcare Bio-Sciences AB (Uppsala, Sweden)
- Mineral oil, Agilent Technologies (Santa Clara, CA, USA)
- 744 pH Meter and Biotrode, Metrohm (Herisau, Switzerland)
- SpeedVac concentrator, Savant SPD111V, Thermo Scientific (Waltham, MA, USA)

Stock solution: Glycerol 50%, 12% (v/v), IPG buffer 0.8%, H_2O 87.2%

Peptide sample solution/ Peptide strip rehydration solution (see note 1)

Stock solution 80%, H_2O 20%

2.7. **LC-MS/MS analysis**

- Acetonitrile (ACN), Chromasolv® for HPLC (≥99.9%), Sigma-Aldrich (Milwaukee, WI, USA)
- Water, Romil-SPS Super purity solvent (≥99.999%), Romil (Cambridge, England)
- Formic acid (FA), ULC/MS grade (99%), Biosolve (Valkenswaard, Netherlands) to be collected with a glass syringe, Hamilton (Bondaduz, Switzerland)
- Easy nLC 1000 system, Thermo Scientific (Waltham, MA, USA)
- Acclaim pepmap100, C18, 3 µm, 75 µm x 20 mm nano trap-column, Thermo Scientific (Waltham, MA, USA)

- Easy-Spray column 75 µm x 500 mm, C18, 2 µm, Thermo Scientific (Waltham, MA, USA)
- LC-ESI-MS/MS on Orbitrap Fusion Lumos Mass Spectrometer, Thermo Scientific (Waltham, MA, USA) (see note 2)

2.8. **Data analysis**

- Proteome Discoverer Software, Thermo Scientific (Waltham, MA, USA) or equivalent software (see note 3)

3. Method

Before starting a TMT analysis, the experimental design should be carefully set-up. Particular attention should be taken to use equal protein concentration in the different samples and to add an internal standard to control the experimental variability within one TMT (see note 4) and/or between several TMT experiments.

3.1. **Sample preparation**

3.1.1. CSF

- Samples can be obtained by lumbar or ventricular puncture following ethically approved clinical protocols (see note 5).
- Filtrate the CSF on Millex-HV filters to remove all membrane and cellular debris (152).
- Depending on the proteins of interest, deplete the CSF from the most abundant proteins (albumin, transferrin, IgG, IgA, α1-antitrypsin and haptoglobin) using MARS column (see note 6).
- Depending on the sample concentration; lyophilize the sample using the speed-vacuum system in order to concentrate it (see note 6).
- Control the protein concentration in the sample by e.g. Bradford assay (see 3.2 Bradford assay).
- The sample can be stored at -80°C until use.

3.1.2. Cerebral tissue

- Cut the frozen tissues in slices of 18 µm (see note 7).
- Homogenize 15 mg of brain tissue in 1mL of solubilisation buffer.
- Perform light sonication; 10 x 60% amplitude, 10 pulse at 0.5s with 1 min on ice between pulses.

- Control the protein concentration in the sample by e.g. Bradford assay (see 3.2 Bradford assay).
- The sample can be stored at -80°C until use.

3.1.3. Neurons

- Trypsinize the brain tissue (0.125% in HBSS, Ca2+- and Mg2+-free) and incubate for 25 min at 37°C (see note 8).
- Wash once with HBSS containing Ca2+and Mg2+ after adding trypsin inhibitor.
- Dissociate the cells in serum-free Neurobasal medium plus B27 supplement.
- Lyse the cells using the lysis buffer.
- Sonicate the lysed neurons.
- Centrifuge the neurons at 20000 g for 15 min at 4°C.
- Extract the proteins from the supernatant using chloroform/methanol (4:1 v/v) (153).
- Re-solubilize the resulting pellet with RapiGest 0.1%.
- Control the protein concentration in the sample by e.g. Bradford assay (see 3.2 Bradford assay).
- The sample can be stored at -80°C until use.

3.2. **Bradford assay**

- Prepare a stock solution of BSA (1µg/µL) by mixing deionized water with lyophilized bovine serum albumin.
- Prepare a dye reactive by mixing 1 part of Dye Reagent Concentrate with 4 parts of deionized water.
- Prepare dilutions for standard curve. The final volume of each tube is 1 mL made of 800 µL sample (water and BSA) and 200 µL reactive. Important: always prepare a blank (BSA concentration = 0 µg/µL).
- In the tube introduce the components in the following order: first H2O, then BSA and lastly the reactive. Mix gently and incubate for 10 minutes at room temperature.
- Prepare samples to test in the same way (e.g. 2 µL sample + 798 µL H2O).
- Just before reading the absorbance at 595 nm, transfer the prepared dilutions into the cuvettes. For more information about how to read the samples, refer to the manufacturer's instructions.
- Graph the standard curve manually or in Excel and calculate the concentration of the tested samples.

3.3. **Reduction, alkylation and protein digestion (see notes 4 and 9)**

- For each tag, take 25 µg proteins from the samples and dry in a speed-vacuum (see note 10).
- Reconstitute the samples using 33 µL of tetraethylammonium bromide (TEAB). TEAB should be prepared in water to obtain TEAB 0.1mM and the pH must be adjusted to 8 by HCl (see note 11).
- Reduce the proteins by adding 2 µL of TCEP to each sample, incubate for 60 min at 37°C and spin down the samples.
- Add 400 mM iodoacetamide to each sample for the alkylation step.
- Incubate in the dark for 30 min at room temperature.
- Add trypsin for a final concentration of 0.2 µg/µL.
- Vortex and spin down the samples.
- Incubate for digestion overnight at 37°C.

3.4. **TMT labeling**

- Leave TMT reagents at room temperature for 15 min.
- Dissolve the lyophilized TMT reagents using 42 µL ACN.
- Leave the reaction under agitation 60 min at room temperature.
- Prepare hydroxylamine 5% (w/v) in water and add 8 µL to each sample.
- Leave the reaction under agitation 15 min at room temperature.
- Pool the samples together in a new tube.
- Clean all the labelled samples tubes 2 x 30 µL H_2O, vortex, spin down and add to the pooled sample tube.
- Dry the pooled sample in a speed-vacuum (see note 12).
- The samples can be stored at - 20 °C until further analysis.

3.5. **Purification (Macro SpinColumn)**

- Take a C18 Macro SpinColumn and place it in an Eppendorf tube.
- Rehydrate the column with 400µl 100% ACN, wait 10min and centrifuge 1min at 1000 g.
- Wash the spin column with 400 µl 50% H_2O+50% ACN+0.1% FA and centrifuge 1min at 1000 g.
- Wash the spin column with 400 µl 95% H_2O+5% ACN+0.1% FA and centrifuge 1min at 1000 g.
- Remove the columns leftovers in the Eppendorf.
- Put 400µl 95% H_2O+5% ACN+0.1% FA into sample to re-suspend it.
- Take 0.3 µl sample to control the pH that should be 3 or slightly below, adjust with 10% FA.

- Place the sample into the column and centrifuge 1min at 1000 g.
- Put 250µl 95% H_2O+5% ACN+0.1% FA in the old sample tube to clean the tube and place it onto the column and centrifuge 1min at 1000 g.
- Wash the column with 400µl 95% H_2O+5% ACN+0.1% FA and centrifuge 1min at 1000 g (see note 13).
- Place the column in a new tube.
- Elute the sample with 300µl 50% H_2O+50% ACN+0.1% FA and centrifuge 1min at 1000 g.
- Dry the sample in speed-vacuum (see note 12).

3.6. **Fractionation (see note 14)**

- Assemble the OFFGEL electrophoresis (OGE) according to manufacturer's recommendations. The following protocol is for 13cm strip - 12 fraction frame (see note 15).
- Dissolve the sample in 1200 µL sample solution and vortex.
- Put 20 µL peptide strip rehydration solution into well 2 to 11, and 40 into well 1 and 12 (see note 16). Gently tap the tray onto the desk, to ensure total rehydration of the strip.
- Wet 4 pads with the rehydration solution. Place two pads on each side of the strip.
- Wait for 30 minutes.
- Vortex and spin the sample.
- Load 150 µL sample solution from well 3 to 10. Add 150 µL peptide sample solution into well 1, 2, 11 and 12 (see note 17).
- Place the cover seal over the frame.
- Place 200 µL oil on the left and 1000 µL on the right side of the frame.
- After 1 minute, add 200 µL oil on each side of the frame.
- Place electrodes on the pads.
- Run the OGE overnight. Set the focusing parameters to; 20Kvh, 8000V, 50µA, 200mW, 100s and the hold parameters to; 500V, 20uA and 50mW.
- Collect each fraction separately. Each cup should be washed with 100 µL of pure water and added to the corresponding sample.
- Control the pH of each fraction to verify the correct separation.
- Dry the samples in speed-vacuum (see note 12).
- The samples can be stored at -20°C for one week until further analysis.

3.7. **Purification (Micro SpinColumn)**

- Take one C18 Micro SpinColumn per OGE fraction and place each one in an Eppendorf tube.

- Rehydrate each column with 200 µl 100% ACN, wait 10min and centrifuge 1min at 700 g.
- Wash spin columns twice with 200 µl 50% H_2O+50% ACN+0.1% FA and centrifuge 1min at 700 g.
- Wash spin columns twice with 200 µl 95% H_2O+5% ACN+0.1% FA and centrifuge 1min at 700 g.
- Remove the columns leftovers in the Eppendorf tube.
- Re-suspend each sample by adding 150 µl 95% H_2O+5% ACN+0.1% FA.
- Take 0.3 µl sample to control the pH that should be 3 or slightly below, adjust with 10% FA. Place one sample per column and centrifuge 1min at 700 g.
- Put 150µl 95% H_2O+5% ACN+0.1% FA in each sample tube to clean the tubes and forward the solution into the corresponding column and centrifuge 1min at 700 g.
- Wash with 200µl 95% H_2O+5% ACN+0.1% FA and centrifuge 1min at 700 g (see note 13).
- Replace the Eppendorf tubes under each column with a new tube.
- Elute the samples twice with 150µL 50% H_2O+50% ACN + 0.1% FA.
- Dry in a speed-vacuum (see note 12) and store the samples at -20°C until mass spectrometry analysis.

3.8. **LC-MS/MS analysis (see notes 18 and 19)**

- Reconstitute the samples in 95% H_2O+5% ACN+0.1% FA for a final sample concentration of ≤ 0.25 µg/µl (see note 20).
- Centrifuge the samples 5 min at 14000 rpm before transferring them into RP-LC vials.
- For the RP-LC, prepare solvent A (H_2O 0.1% FA) and solvent B (ACN 0.1% FA). Run the RP-LC for 85 min using a flow rate of 220 nL/min and with the gradient; 0-1 min 95% A and 5% B, then 65% A and 35% B at 55 min, 20% A and 80% B at 65min for 2 min and re-equilibrate the column at 69 min. The chromatographic system is connected online with the quadruple-Orbitrap spectrometer (Orbitrap Fusion Lumos).
- Analyse the eluted peptides by electrospray ionization (ESI) in positive ion mode (1.9 kV). Use data-dependent acquisition method for mass spectrometry analysis. For the MS1 survey scan, set the Orbitrap resolving power to 120 000 at 200 m/z with a scan range of 300-1500 m/z and an automatic gain control (AGC) value of 400000 (isolation width 0.7 m/z). The most abundant precursor ions in the MS1 scan are selected for subsequent HCD fragmentation with normalized collision energy of 40%. MS/MS spectra are acquired in the Orbitrap analyser with a resolving power of 60000 and dynamic exclusion of 30 seconds to avoid repeated analysis of the same precursor ion (see note 2).

3.9. **Data analysis (see note 21)**

- Upload the .raw files to Proteome Discoverer and chose “fractions” to make the Proteome Discoverer consider all of them as one experiment and not separate analysis (see notes 22 and 23). Add the isotope impurities correction (obtained by Thermo Scientific for each TMT package).
- Chose the processing and consensus workflow with reporter based quantification that match the mass spectrometry instrument used or create your own.
- The processing workflow should include identification by Mascot and percolator to set the peptide FDR to 1% (see note 24). Within Mascot the data are to be searched against a database, e.g. UniProt-Swiss-Prot. Fixed modifications are carbamidomethylation of cysteines, the TMT labelling of peptide amino terminus and lysine (monoisotopic mass modification of +229.162932 Da for TMT 10- and 6-plex) (see note 25). Oxidized methionine is a variable modification. Select trypsin as the used enzyme with the possibility of 1 missed cleavage.
- Parameters to be applied in the consensus workflow are; protein FDR 1% (see note 24), unique and razor peptides, apply the isotopic correction, normalisation (see note 26) and scaling mode.
- Define the tag ratios of interest to be calculated e.g. 126, 127N, 127C, 128N, 128C vs. 129N, 129C, 130N, 130C, 131 (several different ratios can be performed).

4. Notes

1. The same solution is used for rehydration of both sample and strip. However, the solution should be separated in two different tubes in order to avoid sample contamination.
2. Other high resolution mass spectrometry instruments can be used for TMT 10-plex analysis e.g. Orbitrap or Q-TOF. The minimum resolution required is 50000 at 150 m/z. The parameters required may differ from those described here. Different identification and quantification software can be used e.g. Mascot Software Matrix Science, Isobar Bioconductor, Scaffold Q+ Software Proteome Software and MaxQuant software.
3. Internal standard can be used for detection of analytical bias. The samples should be equally spiked using a protein not initially included in the sample e.g. B-Lactoglobulin from bovine when using human samples.
4. Cerebrospinal fluid (CSF) is a body fluid that occupies the subarachnoid space and the ventricular system. The total CSF volume in humans is around 135 mL and it is composed by a few numbers of cells (0-4 cells/µL), low protein concentration (400µg/mL) and salts.

Lumbar puncture is the selected method for the collection. Due to its direct contact with brain it is widely used in neuroproteomics. Protein concentration of CSF from the lumbar region is about 15 to 45 mg/dl protein whereas the protein concentration in cisternal and ventricular collection is lower.

5. The main problem using the CSF for proteomic analysis is the low protein content; therefore it should be suitable to prefractionate it in order to have access to low-abundance proteins using for example antibody depletion. Another solution can be to concentrate the samples.
6. The cellular composition will vary depending on the tissue region. It is important to adjust the protocol depending on the type of sample e.g. cerebral tissue can contain fat that needs to be cleaned (chloroform/methanol precipitation) before use.
7. Neurons have glycolipids, sphingomyelin and cholesterol that make analysis of proteins difficult. The use of detergents or chaotropes among others is optimal for removing these interfering components.
8. The TEAB and trypsin solutions should be freshly prepared for each experiment. The iodacetamide solution should be prepared immediately before use and protected from light.
9. The amount of protein needed per sample will depend on the mass spectrometer used but 25-100 µg are required for proper tagging.
10. Poor labelling may be due to incorrect buffer pH or the use of an amine-based buffer.
11. Add 100µL 95% H_2O+5% ACN+0.1% FA to the dried sample and dry again. This allows getting the proteins that potentially stick on the side of the tube down to the bottom.
12. After adding samples to columns when purifying, the “leftovers” should be kept in case of a poor separation. The purification step can in that case be redone.
13. Before OGE, 5 µL of the pooled sample can be kept and dried for injection in LC-MS/MS for comparison with the OGE results but also as a control for bias search.
14. The OGE can also be performed with 24 fractions.
15. For a complete rehydration of the gel, including the gel situated outside the fractionation frame, more rehydration solution is added in the extremity wells. If not rehydrated enough, the gel might absorb the sample solution and leave the sample dry. Caution should be taken to not rehydrate too much as the current might find another path than through the gel.
16. For an efficient fractionation with the OGE avoid bubbles when pipetting solutions.
17. To avoid PEG contaminants in the samples only use polypropylene tubes and do not store acidic solutions in plastic tubes. The use of gloves also minimizes the contamination risk of PEG coming from soap and hand cream as well as keratins.

18. Remove all detergents that are not MS-compatible e.g. triton, NP-40, SDS, CHAPS and tween.
19. An approximated estimation of the peptide concentration within each fraction can be obtained by compiling the original concentration used for labelling of each tag (e.g. 25 µg/tag * 10 tags) and separate by the number of fractions used in the OGE (250 µg total / 12 fractions).
20. The settings for identification and quantification mentioned here should be considered as "potential guidelines" but adjustments can be necessary depending on sample type, instrument used, software used, experiment design and type of comparison.
21. Depending on identification and quantification software to be used, software to convert the raw files might be necessary. The software MSconvert can be used to convert a number of different files into the required format. The software can be downloaded at the Proteowizard website (http://proteowizard.sourceforge.net/downloads.shtml).
22. Depending on identification and quantification software to be used, the different fractions might need to be merged before use. This can be performed manually by copying all fraction raw files, one after the other, in order to obtain a single file containing all files.
23. The false discovery rate (FDR) at both protein and peptide levels should be set to less than 1% (154).
24. The labelling efficiency can be verified by setting the TMT labelled peptide amino terminus and lysine to be a variable modification. The percentage of non-labelled peptides over the total amount of peptides should be less than 5%.
25. Normalisation can be performed in order to avoid high biological variability. Several normalisation methods exists e.g. the intensity median for each tag is calculated and adjusted to the highest one. The applied factor should not be lower than 0.8.

5. Figures and tables

Figure 1: The TMT 10-plex structure

The position of heavy isotopes, 15N and 13C, are marked with asterisks.

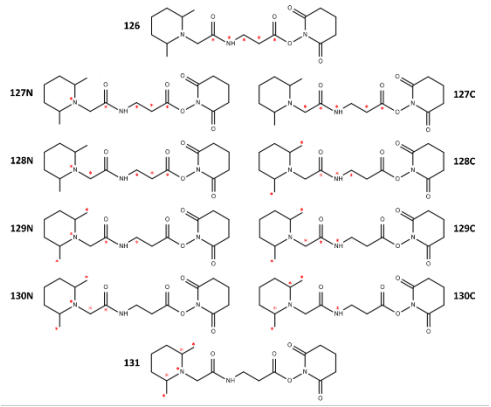
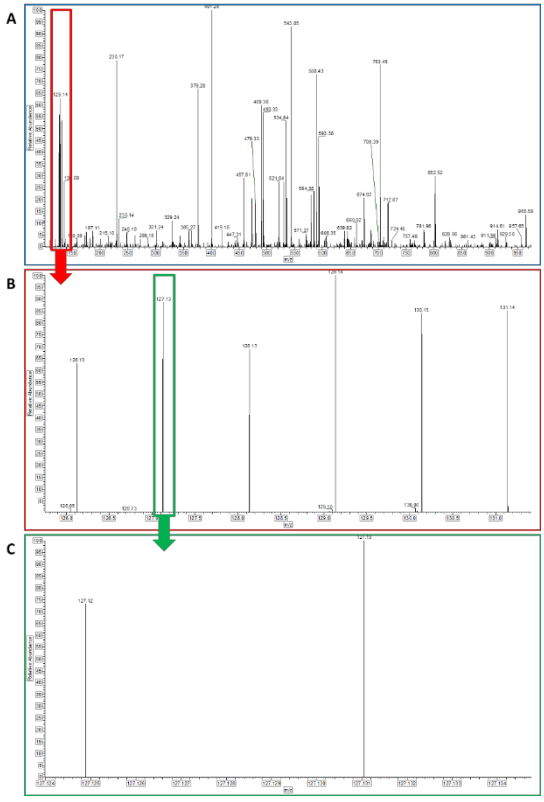


Figure 2: TMT 10-plex spectra MS/MS

HCD fragmented MS/MS spectra including both identification and quantification. A) The MS/MS spectra containing the TMT reporter ions and the peptide ions. B) Zoom on the TMT 10-plex reporter region 126-131 m/z. C) Further zoom on the reporter tags to differentiate between the isotopes e.g. 127N (127.124760 m/z) and 127C (127.131079 m/z).



6. References

1. Shevchenko, G.; Konzer, A.; Musunuri, S.; Bergquist, J., Neuroproteomics tools in clinical practice. *Biochim Biophys Acta* **2015,** 1854, (7), 705-17.

2. Wang, K. K. W.; Montaner, J., Neuroproteomics 101. *Translational Proteomics* **2014,** 3, A1-A2.

3. Craft, G. E.; Chen, A.; Nairn, A. C., Recent advances in quantitative neuroproteomics. *Methods* **2013,** 61, (3), 186-218.

4. Liu, T.; Hu, J.; Li, H., iTRAQ-based Shotgun Neuroproteomics. *Methods in molecular biology (Clifton, N.J.)* **2009,** 566, 201-216.

5. Rauniyar, N.; Yates, J. R., 3rd, Isobaric labeling-based relative quantification in shotgun proteomics. *J Proteome Res* **2014,** 13, (12), 5293-309.

6. Dayon, L.; Turck, N.; Scherl, A.; Hochstrasser, D. F.; Burkhard, P. R.; Sanchez, J. C., From relative to absolute quantification of tryptic peptides with tandem mass tags: application to cerebrospinal fluid. *Chimia (Aarau)* **2010,** 64, (3), 132-5.

7. Thompson, A.; Schafer, J.; Kuhn, K.; Kienle, S.; Schwarz, J.; Schmidt, G.; Neumann, T.; Johnstone, R.; Mohammed, A. K.; Hamon, C., Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal Chem* **2003,** 75, (8), 1895-904.

8. Chiva, C.; Sabido, E., HCD-only fragmentation method balances peptide identification and quantitation of TMT-labeled samples in hybrid linear ion trap/orbitrap mass spectrometers. *J Proteomics* **2014,** 96, 263-70.

9. Dayon, L.; Pasquarello, C.; Hoogland, C.; Sanchez, J. C.; Scherl, A., Combining low- and high-energy tandem mass spectra for optimized peptide quantification with isobaric tags. *J Proteomics* **2010,** 73, (4), 769-77.

10. Ye, H.; Boyne, M. T., 2nd; Buhse, L. F.; Hill, J., Direct approach for qualitative and quantitative characterization of glycoproteins using tandem mass tags and an LTQ Orbitrap XL electron transfer dissociation hybrid mass spectrometer. *Anal Chem* **2013,** 85, (3), 1531-9.

11. Viner, R. I.; Zhang, T.; Second, T.; Zabrouskov, V., Quantification of post-translationally modified peptides of bovine alpha-crystallin using tandem mass tags and electron transfer dissociation. *J Proteomics* **2009,** 72, (5), 874-85.

12. Dayon, L.; Hainard, A.; Licker, V.; Turck, N.; Kuhn, K.; Hochstrasser, D. F.; Burkhard, P. R.; Sanchez, J. C., Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. *Anal Chem* **2008,** 80, (8), 2921-31.

13. Ballard, C.; Gauthier, S.; Corbett, A.; Brayne, C.; Aarsland, D.; Jones, E., Alzheimer's disease. *Lancet* **2011,** 377, (9770), 1019-31.

14. Lv, J.; Ma, S.; Zhang, X.; Zheng, L.; Ma, Y.; Zhao, X.; Lai, W.; Shen, H.; Wang, Q.; Ji, J., Quantitative proteomics reveals that PEA15 regulates astroglial Aβ phagocytosis in an Alzheimer's disease mouse model. *Journal of Proteomics* **2014,** 110, 45-58.

15. Liu, Y.; Qing, H.; Deng, Y., Biomarkers in Alzheimer's disease analysis by mass spectrometry-based proteomics. *Int J Mol Sci* **2014,** 15, (5), 7865-82.

16. Licker, V.; Turck, N.; Kovari, E.; Burkhardt, K.; Cote, M.; Surini-Demiri, M.; Lobrinus, J. A.; Sanchez, J. C.; Burkhard, P. R., Proteomic analysis of human substantia nigra identifies novel candidates involved in Parkinson's disease pathogenesis. *Proteomics* **2014,** 14, (6), 784-94.

17. Dayon, L.; Turck, N.; García-Berrocoso, T.; Walter, N.; Burkhard, P. R.; Vilalta, A.; Sahuquillo, J.; Montaner, J.; Sanchez, J.-C., Brain Extracellular Fluid Protein Changes in Acute Stroke Patients. *Journal of Proteome Research* **2011,** 10, (3), 1043-1051.

18. Laban, K. G.; Rinkel, G. J. E.; Vergouwen, M. D. I., Nosocomial infections after aneurysmal subarachnoid hemorrhage: time course and causative pathogens. *International Journal of Stroke* **2015,** 10, (5), 763-766.

19. Azurmendi, L.; Degos, V.; Tiberti, N.; Kapandji, N.; Sanchez, P.; Sarrafzadeh, A.; Puybasset, L.; Turck, N.; Sanchez, J.-C., Measuring Serum Amyloid A for Infection Prediction in Aneurysmal Subarachnoid Hemorrhage. *Journal of Proteome Research* **2015,** 14, (9), 3948-3956.

20. Nunez Galindo, A.; Kussmann, M.; Dayon, L., Proteomics of Cerebrospinal Fluid: Throughput and Robustness Using a Scalable Automated Analysis Pipeline for Biomarker Discovery. *Anal Chem* **2015,** 87, (21), 10755-61.

21. Yu, L. R.; Conrads, T. P.; Uo, T.; Kinoshita, Y.; Morrison, R. S.; Lucas, D. A.; Chan, K. C.; Blonder, J.; Issaq, H. J.; Veenstra, T. D., Global analysis of the cortical neuron proteome. *Mol Cell Proteomics* **2004,** 3, (9), 896-907.

22. Giron, P.; Dayon, L.; Turck, N.; Hoogland, C.; Sanchez, J. C., Quantitative analysis of human cerebrospinal fluid proteins using a combination of cysteine tagging and amine-reactive isobaric labeling. *J Proteome Res* **2011,** 10, (1), 249-58.

23. Friedman, D. B., Quantitative proteomics for two-dimensional gels using difference gel electrophoresis. *Methods Mol Biol* **2007,** 367, 219-39.

24. Deutsch, E. W.; Overall, C. M.; Van Eyk, J. E.; Baker, M. S.; Paik, Y. K.; Weintraub, S. T.; Lane, L.; Martens, L.; Vandenbrouck, Y.; Kusebauch, U.; Hancock, W. S.; Hermjakob, H.; Aebersold, R.; Moritz, R. L.; Omenn, G. S., Human Proteome Project Mass Spectrometry Data Interpretation Guidelines 2.1. *J Proteome Res* **2016,** 15, (11), 3961-3970.

Chapter III

Neopterin plasma concentrations in patients with aneurysmal subarachnoid hemorrhage: correlation with infection and long-term outcome

Neopterin plasma concentrations in patients with aneurysmal subarachnoid hemorrhage: correlation with infection and long-term outcome

Leire Azurmendi, Vincent Degos, Natalia Tiberti, Natacha Kapandji, Paola Sanchez-Peña, Asita Sarrafzadeh, Louis Puybasset, Natacha Turck, Jean-Charles Sanchez

Infections occurring after aSAH are one of the main factor responsible of outcome worsening and in-hospital deaths. In order to improve the management of the patients, the aim of this chapter was to identify a biomarker able to predict the patients at risk of in-hospital infections as well as their associated outcome.

Based on the literature, the inflammation theory is the key physiopathological pathway leading to deleterious complications, therefore we tested the capacity of neopterin, an already described inflammatory marker, to act as infection and outcome marker in our population of aSAH patients.

Neopterin concentrations were measured in 42 infected and 19 non-infected patients using ELISA kits. Obtained results allowed us to confirm that this metabolite was able to correlate with infection development from three days after hospital admission. The relationship with the outcome of the patients was also highlighted.

This work was published in 2015 in *Journal of neurosurgery*. My contribution consisted in performing the different experiments, data analyses and in writing the paper.

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CLINICAL ARTICLE

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Neopterin plasma concentrations in patients with aneurysmal subarachnoid hemorrhage: correlation with infection and long-term outcome

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OBJECTIVE Aneurysmal subarachnoid hemorrhage (aSAH) is associated with high rates of mortality and morbidity. The main predictor for the poor outcome is the World Federation of Neurosurgical Societies (WFNS) scale. However, this scale does not take into account proinflammatory events, such as infection occurring after the aSAH, which could modify the long-term status of patients. The aim of this study was to evaluate neopterin as an inflammatory biomarker for outcome and infection prediction in aSAH patients.

METHODS Plasma concentrations of neopterin were measured in 61 aSAH patients (22 male and 39 female; mean age [± SD] 52.8 ± 11.8 years) using a commercial ELISA kit. Samples were collected daily for 10 days. Outcome at 12 months was determined using the Glasgow Outcome Scale (GOS) and dichotomized as poor (GOS score 1, 2, or 3) or good (GOS score 4 or 5). Infection was determined by the presence of a positive bacterial culture.

RESULTS Patients with poor outcome at 12 months had higher concentrations of neopterin than patients with good outcome. In the same way, patients who had an infection during the hospitalization had significantly higher concentrations of neopterin than patients without infection ($p = 0.001$). Moreover, neopterin concentrations were significantly ($p < 0.008$) elevated in infected patients 2 days before infection detection and antibiotic therapy.

CONCLUSIONS Neopterin is an efficient outcome predictor after aSAH. Furthermore, it is able to differentiate between infected and uninfected patients as early as 2 days before clinical signs of infection, facilitating earlier antibiotic therapy and better management.

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KEY WORDS aneurysmal subarachnoid hemorrhage; outcome; infection; inflammation; biomarker; neopterin; vascular disorders

Aneurysmal subarachnoid hemorrhage (aSAH) is a subtype of stroke associated with high rates of mortality and morbidity. Fifteen percent of patients die before arriving at a hospital, and 25% of deaths occur within the first 24 hours.[34] Among the survivors, 30% will develop a long-term delayed neurological deficit that will affect their quality of life.[21,32]

The long-term outcome partially depends on early diagnosis and management.[1,12] Therefore, if an aneurysm is detected by CT in a patient with aSAH, invasive treatment is indicated to prevent a second hemorrhage from the affected vessel, the most important cause of death in the first 24 hours following aSAH.[20,25] Large studies, such as the International Subarachnoid Aneurysm Trial (ISAT), have investigated whether neurosurgical clipping or endovascular coiling improve the long-term outcome. It has been

ABBREVIATIONS aSAH = aneurysmal subarachnoid hemorrhage; AUC = area under the ROC curve; DCI = delayed cerebral ischemia; EVD = external ventricular drain; GCS = Glasgow Coma Scale; GOS = Glasgow Outcome Scale; NSE = neuron-specific enolase; ROC = receiver operating characteristic; ROS = reactive oxygen species; S100β = S100 calcium binding protein–β; WBC = white blood cell; WFNS = World Federation of Neurosurgical Societies.

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shown, however, that the outcome depends mainly on unmodifiable factors present at admission and on secondary complications rather than the choice of treatment.[3,20,25]

Until recently, the most accepted criteria for predicting the outcome of aSAH have been increased age and results of clinical and radiological assessment scales for evaluation of the patient's neurological state at hospital admission, such as the Fisher grade, which measures the severity of the hemorrhage,[17] and the World Federation of Neurosurgical Societies (WFNS) scale, which uses the Glasgow Coma Scale (GCS) and assessment of focal neurological deficits to establish the severity of symptoms.[14,17,28]

Interest in the measurement of CSF and blood biomarkers has increased in the last few years because the ability to monitor patients throughout hospitalization may lead to important improvements in their management. Increased concentrations of S100 calcium binding protein–β (S100β), C-reactive protein, adhesion and matrix molecules, and vasogenic and cardiac markers have been found in aSAH patients with poor outcomes, but the limited accuracy of most of these markers has hampered their use in clinical practice.[13,27,36,38]

Neopterin, a catabolic product of GTP (guanosine-5-triphosphate) produced by human monocytes-macrophages upon stimulation with interferon-γ, has been reported as an outcome and infection biomarker for several inflammatory diseases.[6,19,35] Moreover, Mathiesen et al. showed that the CSF and plasma concentrations of neopterin were higher in patients suffering from aSAH than in controls. The use of neopterin to predict patient outcomes was also studied, but no association was found.[23]

We hypothesized that inflammation and infection after the hemorrhagic event have important effects on outcome,[11] and we evaluated neopterin as a biomarker of inflammation and infection that may be useful in predicting outcomes in aSAH patients.

Methods

Patients and Sample Collection

The aSAH patients included in the present study were hospitalized at the Pitié-Salpêtrière Hospital of Paris (France) between July 2004 and April 2008. Aneurysmal SAH was confirmed angiographically and by CT. The study was approved by the local ethics committee (Comité de Protection des Personnes, Pitié-Salpêtrière, Paris, France). All patients or their surrogates signed a written informed consent.

Patient neurological status was evaluated on admission to the intensive care unit by use of the WFNS scale. Depending on the location and the size of the aneurysm, the aneurysm was not treated, was treated by surgical clipping, or was treated by endovascular coil embolization. More details on the clinical monitoring and treatment have been previously described by Turck et al.[36]

Among 198 consecutively hospitalized patients, 137 were excluded from the present study due to 1) presence of more than 1 hemorrhagic event, 2) admission to the hospital more than 48 hours after the onset of symptoms, 3) missing clinical information, or 4) insufficient sample volume to perform our assays. Sixty-one patients were then finally included in this study (Fig. 1).

Plasma samples were collected the day of arrival at the hospital and subsequently every day for 10 consecutive days (Day 1–Day 10).

The Glasgow Outcome Scale (GOS) was used to assess patient outcomes by telephone interview 12 months after the hemorrhagic event. Depending on the grade of dependence, the outcome was classified as poor (GOS score 1, 2, or 3) or good (GOS score 4 or 5) by a doctor who had not participated in the initial care.

During hospitalization, patient infection status was defined daily based on the criteria established by the Interna-

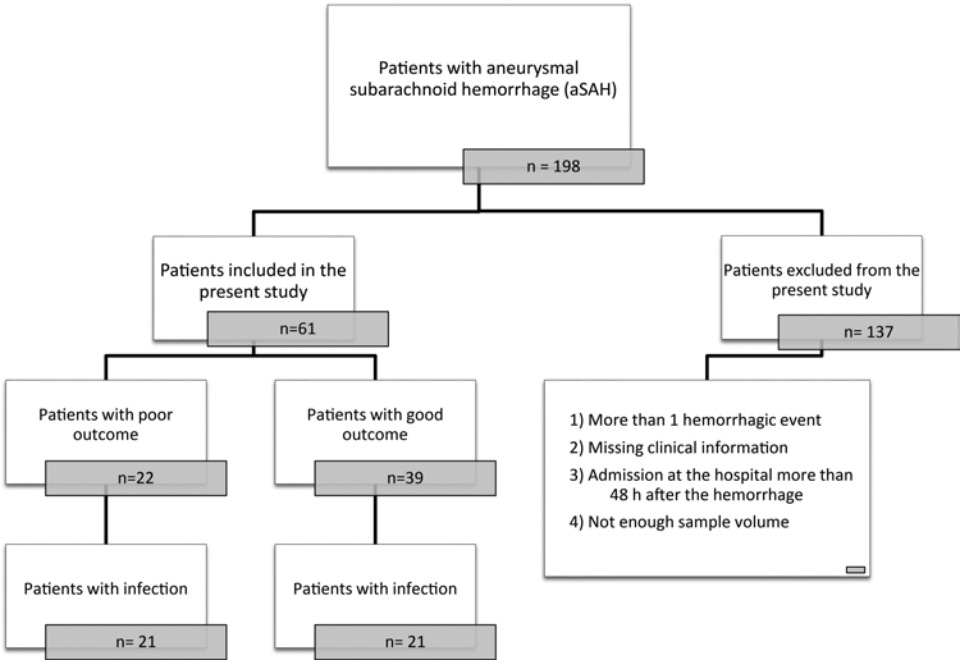

FIG. 1. Inclusion criteria flowchart for the population investigated in this study.

tional Sepsis Forum Consensus Conference on Definitions of Infection in the Intensive Care Unit.[4] When there was a suspicion of infection based on systematic clinical and biological criteria, bacteriological samples were obtained. Antibiotic therapy was started as soon as there was suspicion of infection and was readjusted once the results of the cultures were obtained. The first day of antibiotic therapy in infected patients was designated as T–0, the day before treatment as T–1, and 2 days before treatment as T–2.

Neopterin and S100β Assays

Neopterin concentrations in plasma samples were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (BRAHMS GmbH).

S100β concentrations were determined with an immunoluminometric sandwich assay on an LIA-mat 300 analyzer (Byk-Sangtek France Laboratories) using manufacturer's reagents.[2] Technical variability was assessed using internal quality controls (coefficient of variation ≤ 20%).

Concentrations of both molecules were measured once per day during the first 10 days of hospitalization in order to evaluate their capacity to act as outcome and infection markers.

Statistical Analysis

Statistical analyses were performed with SPSS software (version 21, SPSS Inc.). The Shapiro-Wilk test was used to test deviations from Gaussian distribution of age, neopterin concentration, and S100β concentration. Age was normally distributed, and a parametric test was performed to compare the differences between the ages of patients with good versus poor outcome. Because neopterin and S100β concentrations did not follow a normal distribution, the Mann-Whitney U-test was used for statistical comparisons between 2 unpaired groups and the Friedman test for comparisons of paired groups. Fisher's exact test and the chi-square test were used to assess whether the groups of patients with good or poor outcome differed significantly with respect to sex, WFNS grade, modified Fisher scale grade, presence of convulsions, use of external ventricular drain (EVD), presence of hydrocephalus, and treatment. Univariate and multivariate analyses were performed to assess the association between variables. In a first analysis, the 12-month GOS score was set as the dependent variable and the WFNS grade, S100β concentration, neopterin concentration, and presence of infection as confounders. In a second analysis, the presence/absence of infection was set as the dependent variable and sex, WFNS grade, blood white blood cell (WBC) count, neopterin concentration, and EVD use were set as confounders. The model was validated using the bootstrap method. Categorical data were dichotomized according to the criteria of the demographic characteristics table. Longitudinal data were also dichotomized according to the best cutoff obtained from area under the receiver operating characteristic (ROC) curve (AUC) analysis.

All statistical tests were bilateral, and a p value < 0.05 was considered statistically significant. When multiple comparisons were done, Bonferroni correction of the p values was applied. Sample size estimation was calculated to obtain a power of 90% and a 2-sided error of 5%.[30]

Receiver operating characteristic curves were calculated for WFNS grade at Day 1 and for neopterin and S100β concentrations from Day 1 to Day 10. AUC values, specificity, sensitivity, and 95% CIs were calculated with the pROC package for S+, version 8.1. (TIBCO Software Inc.).[30] A cutoff value corresponding to the best combination of specificity and sensitivity was obtained at each time point for both markers. The Youden index (J) was obtained using the following formula: J = (SE% + SP%) – 100, where SE is sensitivity and SP is specificity.

Results

Demographic Characteristics

The demographic characteristics of the 61 patients included in this study are summarized in Table 1. Most of the patients were women (64%), and the patients' mean age (± SD) was 52.8 ± 11.8 years. In this cohort, 61% of the patients had no motor deficit at admission to the hospital (WFNS grade 1 or 2), and 75% were treated with embolization. Angiographic vasospasm developed in 38% of the patients and infection in 69%.

Prediction of Outcome According to Neopterin and S100β Plasma Concentrations

At 12 months after hospital admission there were no significant differences between patients with good and those with poor outcomes with respect to the modified Fisher scale grade or the development of delayed cerebral ischemia (DCI), seizures (data not shown), or angiographic vasospasm. Nevertheless, univariate analyses showed that even though our study group of patients with aSAH included more women than men, the number of men who had a poor outcome (n = 12, 54.5%) was greater than the number of women (n = 10, 45.5%). WFNS grade and GCS score were highly associated with poor outcome at 12 months ($p < 0.0001$). Furthermore, the intervention technique (clipping or coiling) appeared to play an important role in the long-term outcome, as all the patients whose aneurysms were not treated had a poor outcome at 12 months ($p = 0.005$). Finally, most of the patients with a poor outcome after hospitalization (21 of 22) developed an infection during hospitalization, which strengthened the association between outcome and the presence of infection ($p = 0.007$, OR 18).

To evaluate the utility of neopterin and S100β for predicting 12-month outcome after aSAH, the concentrations were measured every day. Patients were classified according to poor (GOS score 1, 2, or 3) or good (GOS score 4 or 5) outcome at 12 months. Results are shown in Fig. 2. Patients with poor outcomes showed significantly higher plasma concentrations of neopterin and S100β than patients with good outcome. The S100β concentrations were elevated from the day of hospital admission and remained elevated during the whole period of measurement, whereas significantly higher concentrations of neopterin were found from 3 days after hospitalization (Day 3) onward. Moreover, in the case of neopterin, a progressive increase in concentrations was observed.

TABLE 1. Comparison of demographic and clinical characteristics of 61 aSAH patients stratified by outcome at 12 months*

Characteristic	Total (n = 61)	Outcome at 12 Mos		p Value†
		Good (n = 39)	Poor (n = 22)	
Sex				**0.03**
Male	22 (36.1%)	10 (25.6%)	12 (54.5%)	
Female	39 (63.9%)	29 (74.4%)	10 (45.5%)	
Age in yrs				0.875‡
Mean (SD)	52.8 (11.8)	52.3 (12.5)	53.8 (10.5)	
WFNS grade				**<0.0001**
1 or 2	37 (60.7%)	32 (82.1%)	5 (22.7%)	
3, 4, or 5	24 (39.3%)	7 (17.9%)	17 (77.3%)	
GCS score				**<0.0001**
<9	15 (24.6%)	2 (5.1%)	13 (59.1%)	
9–12	7 (11.5%)	3 (7.7%)	4 (18.2%)	
≥13	39 (63.9%)	34 (87.2%)	5 (22.7%)	
Vasospasm				0.871
Yes	23 (37.7%)	15 (38.5%)	8 (36.4%)	
No	38 (62.3%)	24 (61.51%)	14 (63.6%)	
Treatment				**0.005**
Surgery	11 (18%)	5 (12.8%)	6 (27.3%)	
Embolization	46 (75.4%)	34 (87.2%)	12 (54.5%)	
No treatment	4 (6.6%)	0 (0%)	4 (18.2%)	
EVD				**<0.0001**
Yes	37 (60.6%)	16 (41 %)	21 (95.5%)	
No	24 (39.4%)	23 (59%)	1 (4.5%)	
Infection				**<0.0001**
Yes	42 (68.9%)	21 (53.8%)	21 (95.5%)	
No	19 (31.1%)	18 (46.2%)	1 (4.5%)	

* Data are numbers of patients (%) unless otherwise indicated. Good outcome represents a GOS score of 4 or 5; poor outcome, a GOS score of 1, 2, or 3.

† Based on Fisher's exact test or chi-square test, except where otherwise indicated. Bold type indicates statistical significance.

‡ Based on Mann-Whitney U-test.

To evaluate the performance of these 2 variables for the prediction of patient outcomes, ROC curves were used. Detailed results are shown in Table 2 and Figs. 3 and 4. For neopterin, the best discrimination was found 5 days after hospitalization: AUC 84.2% (95% CI 71.9%–94.1%) for a cutoff concentration of 12.7 nmol/L, with specificity and sensitivity values of 74.4% and 86.4%, respectively (J = 60.8). For S100β, the best discrimination was found 10 days after hospitalization: AUC 86.9% (95% CI 73.5%–97.3%) for a cutoff concentration of 0.2 μg/L, with specificity and sensitivity values of 86.4% and 88.2%, respectively.

Taking into account the best cutoff values obtained from the ROC analysis (AUC), we dichotomized the longitudinal variables in univariate and multivariate regression analyses. Univariate analysis showed that sex, presence of infection, and WFNS grade, as well as the concentrations of neopterin and S100β, were associated with outcome at 12 months. In multivariate analysis, however, only neopterin concentration and WFNS grade displayed a relationship with the patients' long-term status (Table 3).

The combination of WFNS grade at admission to the hospital—AUC 89% (95% CI 81.3%–96.7%), specificity 87.2% and sensitivity 77.3% (J = 64.5)—and neopterin concentration at Day 5 dramatically improved the overall accuracy of outcome prediction, reaching a Youden index (J) of 75.5.

Infection Prediction According to Plasma Neopterin Concentration and WBC Count

Infection in aSAH patients can produce systemic inflammatory response syndrome, with associated elevated body temperature, elevated heart rate, tachypnea, and leukocytoses. Because these symptoms appeared to be important factors affecting prognosis after aSAH (p = 0.001), we evaluated whether WBC counts and neopterin concentrations were significantly different in patients with (n = 42) and without infection (n = 19).

We first evaluated the capacity of these variables to help us decide whether or not antibiotic therapy should be initiated. We found that the concentrations of neopterin obtained on the first day of antibiotic treatment for patients who developed an infection were significantly greater than concentrations obtained at the corresponding time point

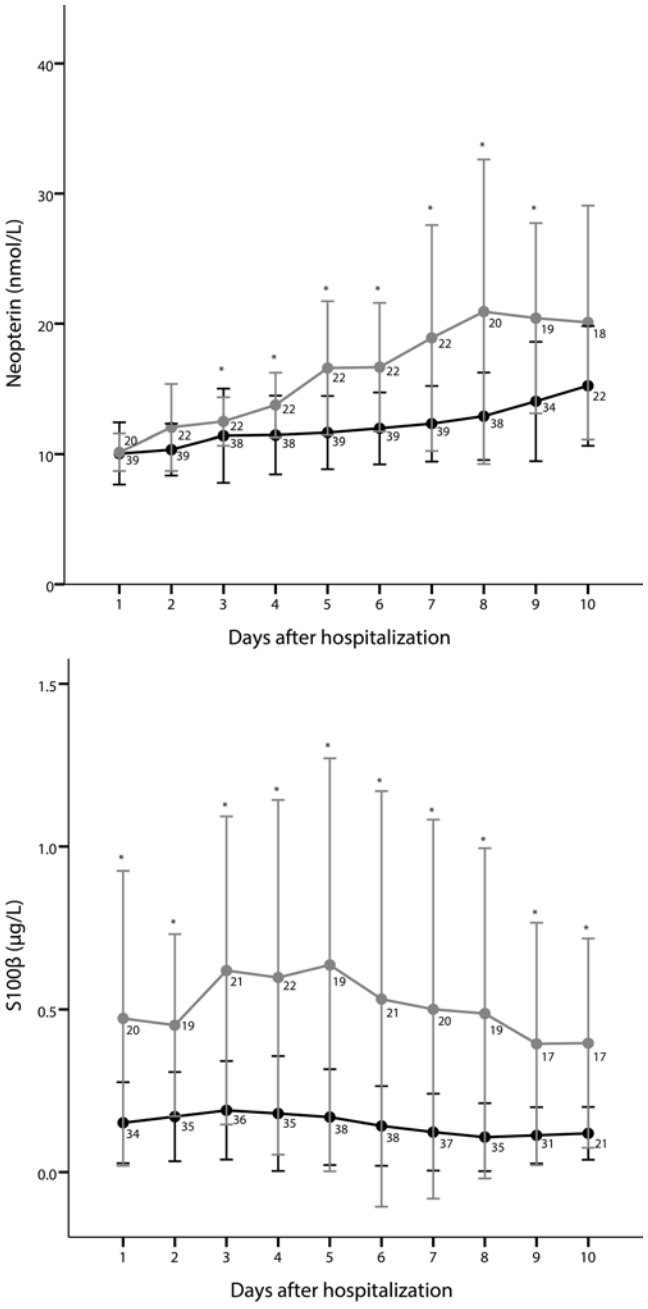

FIG. 2. Kinetics of neopterin **(upper)** and S100β **(lower)** concentrations from the day of hospital admission to 10 days thereafter with patients grouped according to outcome. The mean neopterin and S100β concentrations are indicated by *solid black circles* for the patients with poor 12-month outcome (GOS score 1–3) and by *solid gray circles* for those with good outcome (GOS score 4 or 5). *Error bars* in corresponding colors represent the standard deviations. The numbers on the graphs represent the number of patients tested at each time point. Comparison between 2 groups was performed using the Mann-Whitney U-test. *p < 0.05, after Bonferroni correction.

in those who did not develop an infection (see *Discussion*) (Fig. 5). The Youden index performance, used to differentiate between these 2 groups, reached a value of J = 39.59. In contrast, the WBC count did not differ significantly between the 2 groups (see Table 5 and Fig. 6).

Because neopterin concentration appeared to be an efficient biomarker for infection, we evaluated its performance throughout the entire hospitalization. Neopterin concentrations were significantly higher in infected patients from 3 days after hospitalization onward up to 10 days (Fig. 7). The performance of this biomarker in establishing this differentiation was most effective at Day 3 (AUC 76.7%), which corresponded in most of the cases with 2 days before the start of infection treatment (Table 5 and Fig. 8).

To evaluate whether this increase in neopterin concentrations in infected patients was correlated with the appearance of infection, univariate analyses were performed using values obtained at Day 5, which represented the mean day of infection detection (i.e., the mean of the numbers of days of hospitalization at which infection was detected). Univariate analysis demonstrated that there was a relationship between the presence of an infection and high concentrations of neopterin, poor neurological state at admission to the hospital, and the placement of an EVD. There was, however, no relation between the WBC count and the development of infection. When we tested the same parameters in multivariate analyses, neopterin remained an independent factor for the presence of infection in aSAH patients (Table 4).

Discussion

Long-term outcome after aSAH is a major clinical issue, and the rates of mortality and morbidity are high.[31] The prediction of 12-month outcome as well as the detection of complications during the hospitalization may help physicians to provide better care for aSAH patients and better inform family members of what to expect during the months to come.[15]

Several unmodifiable factors, such as increased age or the level of consciousness of the patient at hospital admission, have been proposed and used as the best univariate predictors of outcome.[17] Among these, we found that the level of consciousness (GCS score) and focal neurological deficit (WFNS score) were the strongest predictors ($p < 0.001$). However, the failure of these neurological assessment scales to address complications occurring during hospitalization has highlighted the need for blood biomarker identification.

The calcium-binding protein S100β has been one of the most commonly used prognostic biomarkers in various conditions associated with brain damage, such as traumatic brain injury, stroke, and aSAH.[27] However, the use of this biomarker in clinical practice has been impeded by limitations in its sensitivity and specificity. Mathiesen et al. evaluated the capacity of neopterin to act as a biomarker for outcome prediction in aSAH patients, but no association was found between neopterin concentrations and patient outcomes.[23] In the present study, however, we found that increased concentrations of neopterin predicted poor outcome at 12 months in 61 patients followed for 10 days after aSAH. Although the concentrations of neopterin followed the same kinetics in both studies, the low number of patients included in the prior analysis may have been the cause of this difference in outcome determina-

TABLE 2. Capacity of plasma concentrations of neopterin and S100β and WFNS grade to discriminate between patients with good and poor outcome

Variable & Day*	No. of Patients		Mean Concentration		p Value†	ROC Curve				
	Good Outcome	Poor Outcome	Good Outcome	Poor Outcome		AUC (95% CI)	Cutoff	SP % (95% CI)	SE % (95% CI)	J
Neopterin (nmol/L)										
D1	39	20	10.04 ± 2.4	10.13 ± 1.4	0.212	60.1 (44.5–75)	10.2	76.9 (64.1–89.7)	50 (30–70)	26.9
D2	39	22	10.34 ± 1.9	12.03 ± 3.3	0.008	70.6 (56.2–83.3)	9.8	48.7 (33.3–64.1)	90.9 (77.3–100)	39.6
D3	38	22	11.39 ± 3.6	12.49 ± 1.9	0.004	72.6 (59.2–84.6)	10.8	57.9 (42.1–73.9)	90.9 (77.3–100)	48.8
D4	38	22	11.45 ± 3	13.75 ± 2.5	0.001	77.03 (64.5–88)	10.9	57.9 (42.1–73.7)	90.9 (77.3–100)	48.8
D5	39	22	11.64 ± 2.8	16.59 ± 5.1	<0.0001	84.2 (71.9–94.1)	12.7	74.4 (58.9–87.2)	86.4 (72.7–100)	60.8
D6	39	22	11.96 ± 2.7	16.66 ± 4.9	<0.0001	81.4 (70.3–90.9)	12	61.5 (46.2–76.9)	95.5 (86.4–100)	57
D7	39	22	12.32 ± 2.9	18.91 ± 8.7	<0.0001	84.2 (73.2–92.8)	12.1	58.9 (43.6–74.4)	95.5 (86.4–100)	54.4
D8	38	20	12.89 ± 3.3	20.93 ± 11.7	<0.0001	84.3 (73.7–93.2)	13.3	65.8 (50–81.6)	90 (75–100)	55.8
D9	34	19	14.03 ± 4.6	20.42 ± 7.3	<0.0001	79.3 (65.9–90.4)	13.7	58.8 (41.2–76.5)	94.7 (84.2–100)	53.5
D10	22	18	15.23 ± 4.6	20.09 ± 8.9	0.058	68.6 (49.8–83.08)	22.9	100 (100–100)	33.3 (11.1–55.6)	33.3
S100β (μg/L)										
D1	34	20	0.15 ± 0.12	0.47 ± 0.45	<0.0001	79.9 (65.9–91.5)	0.3	91.2 (79.4–100)	60 (40–80)	51.2
D2	35	19	0.17 ± 0.14	0.45 ± 0.28	<0.0001	84.4 (72.8–93.5)	0.1	57.1 (40–74.3)	100 (100–100)	57.1
D3	36	21	0.19 ± 0.15	0.63 ± 0.47	<0.0001	81.61 (68.4–92.5)	0.3	86.1 (75–97.2)	66.7 (47.6–85.7)	52.8
D4	35	22	0.18 ± 0.18	0.59 ± 0.54	<0.0001	80.13 (67.8–90.5)	0.2	65.7 (48.6–80)	86.4 (72.7–100)	52.1
D5	38	19	0.17 ± 0.15	0.64 ± 0.44	<0.0001	78.7 (64.6–90.4)	0.4	87.5 (76.3–97.4)	55.6 (36.8–78.9)	44.7
D6	38	21	0.14 ± 0.12	0.53 ± 0.64	<0.0001	82.5 (70.6–92.4)	0.2	71.1 (55.3–84.2)	80.9 (61.9–95.2)	52
D7	37	20	0.12 ± 0.12	0.5 ± 0.40	<0.0001	84.9 (73.9–93.9)	0.2	81.1 (67.6–91.9)	75 (55–90)	56.1
D8	35	19	0.11 ± 0.10	0.49 ± 0.50	<0.0001	84.6 (72.5–94.4)	0.2	91.4 (80–100)	73.7 (52.6–89.5)	65.1
D9	31	17	0.11 ± 0.10	0.39 ± 0.37	<0.0001	85.01 (71.7–95.5)	0.2	90.3 (77.4–100)	76.5 (52.9–94.1)	66.8
D10	21	17	0.12 ± 0.10	0.4 ± 0.32	<0.0001	86.9 (73.5–97.3)	0.2	86.4 (71.4–100)	88.2 (70.6–100)	73.9
WFNS grade										
D1	39	22	1.9 ± 1.2	4 ± 1.2	<0.0001	89 (81.3–96.7)	3.5	87.2 (76.9–87.2)	77.3 (59.1–90.9)	64.5

J = Youden index; SE = sensitivity; SP = specificity.

* Day refers to the number of days after hospitalization.

† Mann-Whitney U-test.

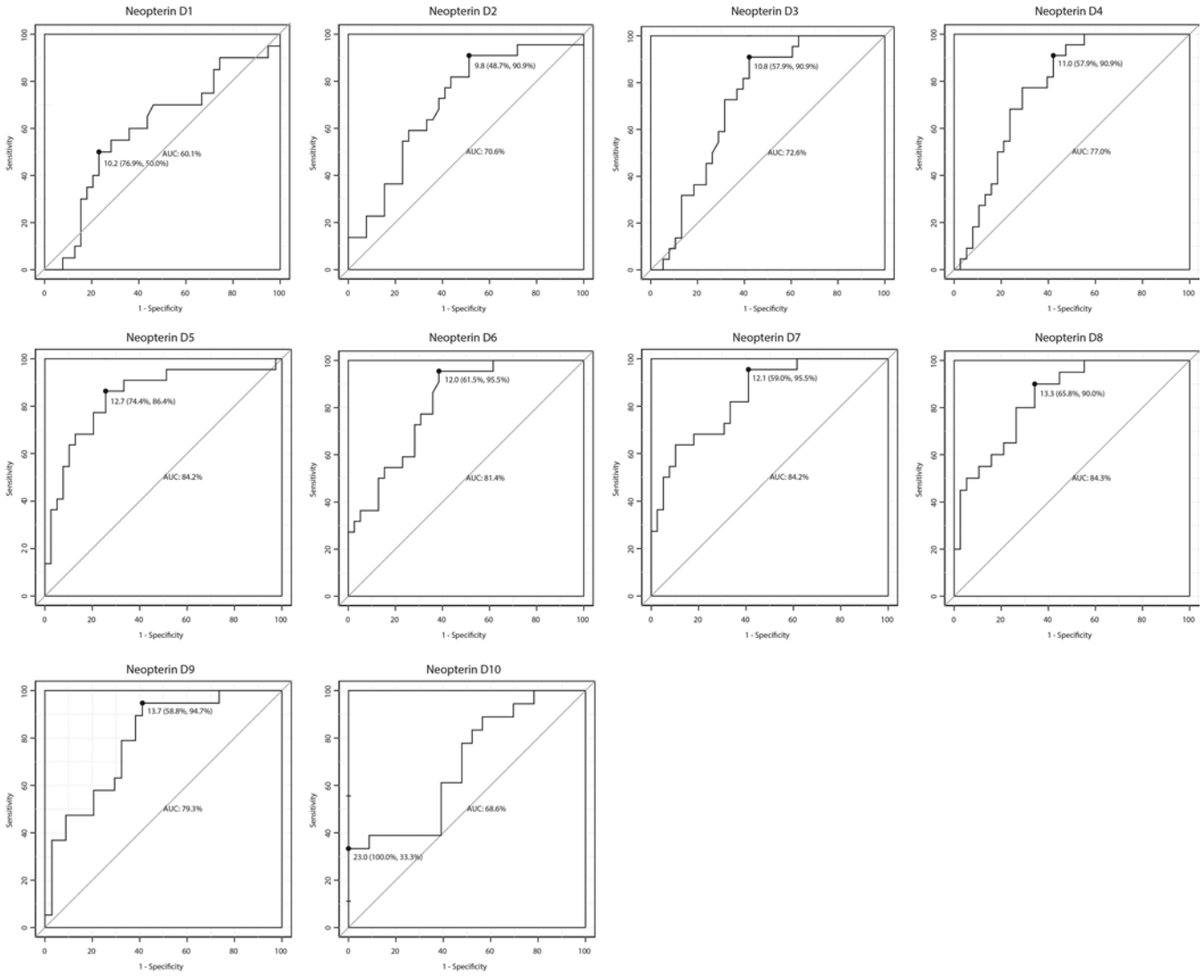

FIG. 3. ROC curves for neopterin representing the ability to differentiate between patients with poor and those with good outcomes from the day of hospital admission (Day 1 [D1]) through the 10th day of hospitalization.

tion. The performance of neopterin in differentiating between patients with good and poor outcomes is quite similar to that of S100β or WFNS. Nevertheless, the combination of neopterin with WFNS data substantially increases the total values for sensitivity and specificity, reaching a Youden index value (J) of 75.5. This fact highlights the finding that although the impact of initial hemorrhage is a key factor affecting outcome, monitoring the patient during the acute phase is also important for improving the patient's outcome.

There are 2 major advantages of neopterin over already established prognostic methods. First, neopterin is an earlier biomarker than S100β, making it possible to identify, only 5 days after hospitalization, patients who are at higher risk of inflammatory complications. This allows the application of treatment before complications appear, increasing the level of care and consequently decreasing the long-term deterioration risk.

The second advantage is that neopterin can function as a biomarker for complications such as infection or DCI that are not addressed by the currently used clinical scales. These events must be considered during the process of biomarker discovery in aSAH, because they appear to be the main causes of pathophysiological worsening.

After the hemorrhagic event, the blood clot in the subarachnoid space leads to recruitment of adhesion molecules at the surface of endothelial cells.[5,22,24,29] Immunological cells such as neutrophils and macrophages phagocytize the red blood cells and degranulate between 2 and 4 days after activation, releasing a large quantity of intracellular endothelins and reactive oxygen species (ROS).[26] The release of ROS causes a decrease in vasodilatation, leading to cerebral vasoconstriction and the development of angiographic vasospasm and DCI.[37] Because vasospasm and DCI are associated with important adverse effects on quality of life, many studies have been conducted to investigate their underlying mechanisms. However, other inflammatory events that play important roles in the long-

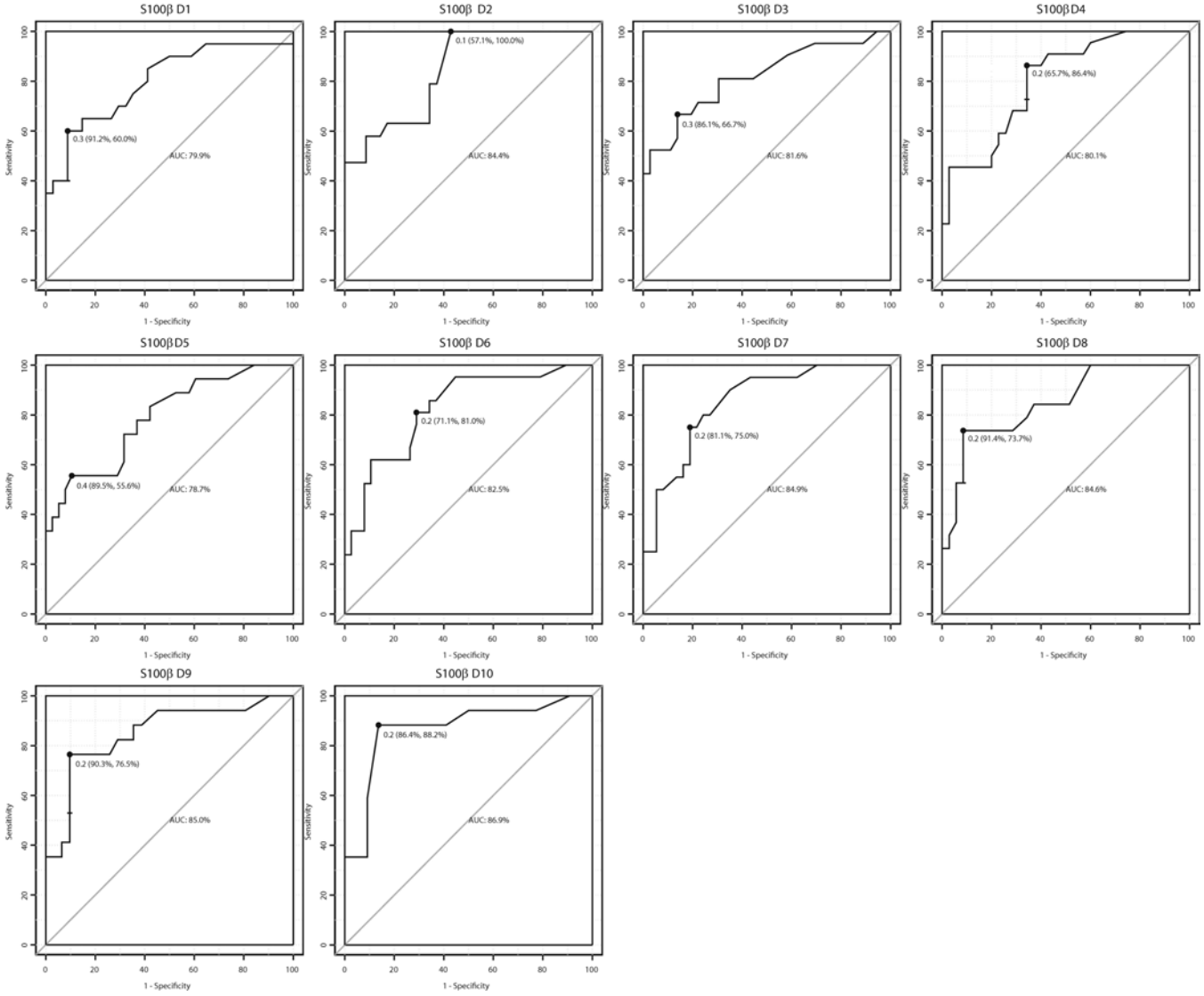

FIG. 4. ROC curves for S100β representing the ability to differentiate between poor and good outcome patients from the day of hospital admission (Day 1 [D1]) through the 10th day of hospitalization.

TABLE 3. Univariate and multivariate analysis of different factors for predicting outcome at 12 months after aSAH*

Analysis & Factor	p Value	OR (95% CI)
Univariate analysis		
Sex	**0.027**	0.287 (0.09–0.87)
WFNS grade	**<0.0001**	15.54 (4.28–56.44)
S100β (<0.4 μg/L)	**0.026**	3.71 (1.17–11.8)
Neopterin (12.7 nmol/L)	**<0.0001**	18.37 (4.46–75.52)
Infection	**0.007**	17.8 (2.2–142.7)
Multivariate analysis		
WFNS grade	**0.001**	12.91 (2.79–59.68)
Neopterin (12.7 nmol/L)	**0.001**	15.34 (3.03–77.77)

* Data obtained at Day 5 in a total of 61 cases were analyzed. The dichotomization of longitudinal data was made according to the cutoff obtained in the AUC analysis at Day 5; the applied values are written next to the variable. Bold type indicates statistical significance.

TABLE 4. Univariate and multivariate analysis of different factors for predicting the presence of infection*

Analysis & Factor	p Value	OR (95% CI)
Univariate analysis		
Sex	0.29	0.53 (0.16–1.73)
WFNS grade	**0.004**	21.8 (2.66–178.53)
WBC (10.9×10^6 cells/mm)	0.18	2.75 (0.63–12.1)
Neopterin (12.7 nmol/L)	**0.002**	8.66 (2.17–34.49)
EVD	**<0.0001**	13.75 (3.65–51.81)
Multivariate analysis		
WFNS grade	0.099	6.88 (0.69–68.29)
Neopterin (12.7 nmol/L)	**0.03**	5.63 (1.13–28.06)
EVD	**0.01**	6.97 (1.55–31.37)

* Data obtained at Day 5 in a total of 61 cases were analyzed. The dichotomization of longitudinal data was made according to the cutoff obtained in the AUC analysis at Day 5; the applied values are written next to the variable. Bold type indicates statistical significance.

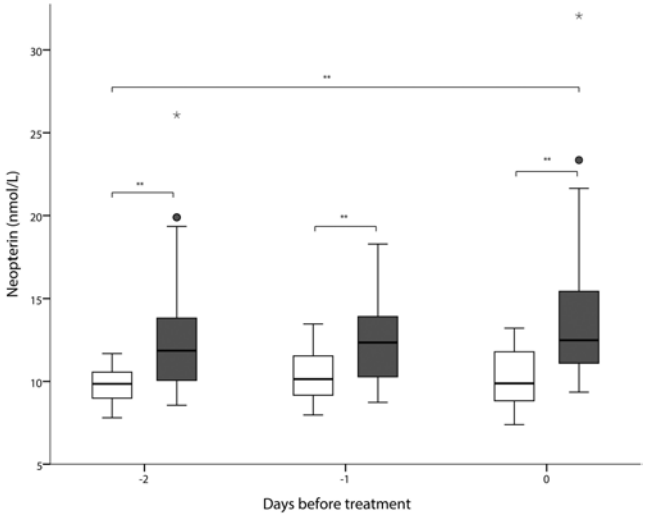

FIG. 5. Box-and-whiskers plots showing the concentrations of neopterin 2 days before initiation of antibiotic therapy, 1 day before initiation of antibiotic therapy, and the day that antibiotic therapy was initiated, with patients stratified by the presence (*gray boxes*, n = 35) or absence (*white boxes*, n = 16) of infection. The *boxes* represent interquartile ranges, the *dark horizontal lines* represent medians, the *whiskers* represent the adjacent values (Q1 – [1.5 × IQR] to Q3 + [1.5 × IQR], where IQR is interquartile range), and the outliers are shown by *solid circles*. Comparisons between the 2 groups were performed using the Mann-Whitney U-test. The Friedman test and Dunn posttest were used to compare the mean rank concentrations of neopterin 2 days before treatment and the day of treatment. Significance is reported after Bonferroni correction. *p < 0.05, **p < 0.01, ***p< 0.001.

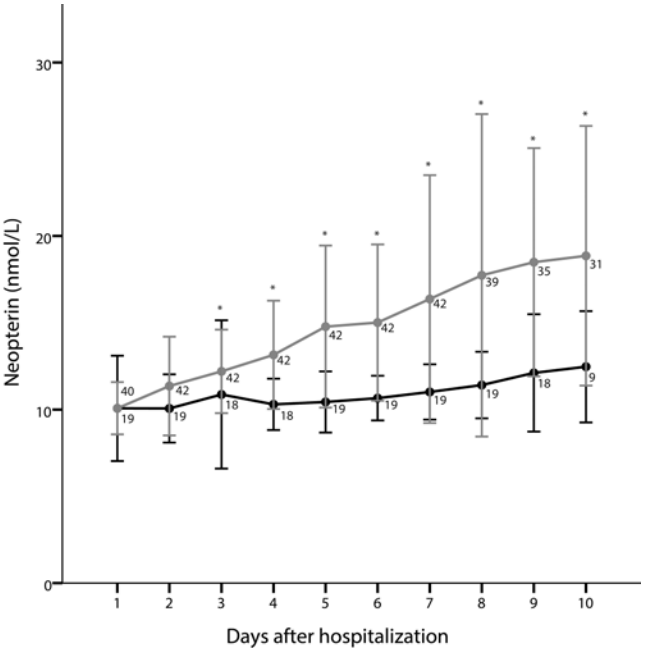

FIG. 7. Kinetics of neopterin concentration from the day of hospital admission to Day 10, with patients grouped according to the presence or absence of infection. The mean neopterin concentration is shown by *solid black circles* for the patients who had infection and by *solid gray circles* for those without infection. *Error bars* in corresponding colors represent the standard deviations. The numbers on the graphs represent the number of patients tested at each time point. Comparison between 2 groups was performed using the Mann-Whitney U-test. *p < 0.05, after Bonferroni correction.

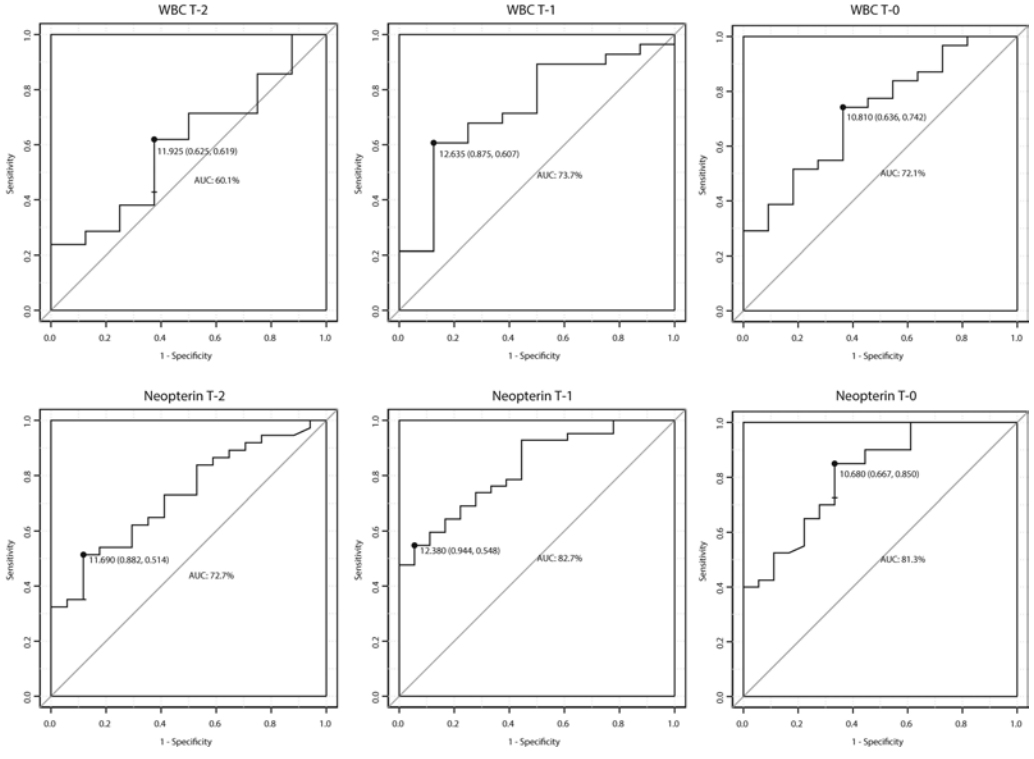

FIG. 6. ROC curves for neopterin and WBC representing the ability to differentiate between infected and uninfected patients the day antibiotic therapy was initiated (T–0), 1 day before (T–1), and 2 days before (T–2).

TABLE 5. Capacity of neopterin and WBC to discriminate between patients with and without infection in plasma samples at different time points

Biomarker & Time Point*	No. of Patients		Mean Concentration ± SD		p Value†	ROC Curve				
	No Infection	Infection	No Infection	Infection		AUC % (95% CI)	Cutoff‡	SP % (95% CI)	SE % (95% CI)	J
Neopterin (nmol/L)										
T–2	17	37	9.99 ± 2.55	12.57 ± 3.72	0.008	72.7 (58.8–85.5)	11.69	88.2 (70.6–100)	51.4 (35.1–67.6)	39.59
T–1	18	42	10.22 ± 1.26	13.05 ± 3.3	<0.0001	82.7 (71–92.1)	12.38	94.4 (83.3–100)	54.7 (40.5–69.1)	49.2
T–0	18	40	10.24 ± 1.84	14.04 ± 4.5	<0.0001	81.3 (68.9–91.7)	10.68	66.7 (44.4–88.89)	85 (72.5–95)	51.67
WBC (10^6 cells/mm)										
T–2	8	21	11.07 ± 3.33	13.67 ± 4.61	0.429	60.1 (36.9–81.6)	12	62.5 (25–87.5)	61.9 (42.9–81)	24.4
T–1	8	28	10.57 ± 3.2	14.61 ± 6.01	0.044	73.7 (52.7–91.1)	12.6	87.5 (62.5–100)	60.71 (42.9–78.6)	48.21
T–0	11	31	10.36 ± 2.84	14.32 ± 4.87	0.03	72.1 (53.4–87.7)	10.8	63.6 (36.4–90.9)	74.19 (58.1–90.3)	37.83
Neopterin (nmol/L)										
D1	19	40	10.1	10.1	0.253	59.28 (43.2–75.4)	9.55	63.2 (42.1–84.2)	57.5 (42.5–72.5)	20.7
D2	19	42	10.1	11.4	0.03	67.5 (52.92–82.04)	9.78	57.9 (36.8–78.9)	76.2 (61.9–88.1)	34.1
D3	18	42	10.9	12.2	0.001	76.7 (63.17–90.27)	11.76	88.9 (72.2–100)	59.5 (45.2–73.8)	48.4
D4	18	42	10.3	13.2	0.001	78.2 (66.6–89.75)	11.54	83.3 (66.7–100)	66.7 (52.4–80.9)	50
D5	19	42	10.4	14.8	<0.0001	83.3 (73.15–93.52)	12.1	84.2 (68.4–100)	71.4 (57.1–83.3)	55.6
D6	19	42	10.7	15	<0.0001	84.8 (75.13–94.42)	12.3	94.7 (84.2–100)	76.2 (61.9–88.1)	70.9
D7	19	42	11	16.4	<0.0001	83.9 (74.02–93.78)	12.3	84.2 (68.4–100)	78.6 (66.7–90.5)	62.8
D8	19	39	11.4	17.7	<0.0001	85.1 (75.25–94.93)	14.24	94.7 (84.2–100)	69.2 (53.9–84.6)	63.9
D9	18	35	12.1	18.5	<0.0001	84.4 (72.7–96.2)	13.8	88.9 (72.2–100)	82.9 (68.6–94.3)	71.8
D10	9	31	12.5	18.9	0.003	82.1 (67.3–96.9)	14.8	88.9 (66.7–100)	70.9 (54.8–87.1)	59.8

* T–2, T–1, and T–0 refer, respectively, to 2 days before, 1 days before, and the day of initiation of antibiotic therapy, while D1–D10 refer to Day 1 through Day 10 of hospital stay.

† Based on Mann-Whitney U-test with Bonferroni correction.

‡ The reported cutoff corresponds to the best combination of specificity and sensitivity obtained for each time point and biomarker.

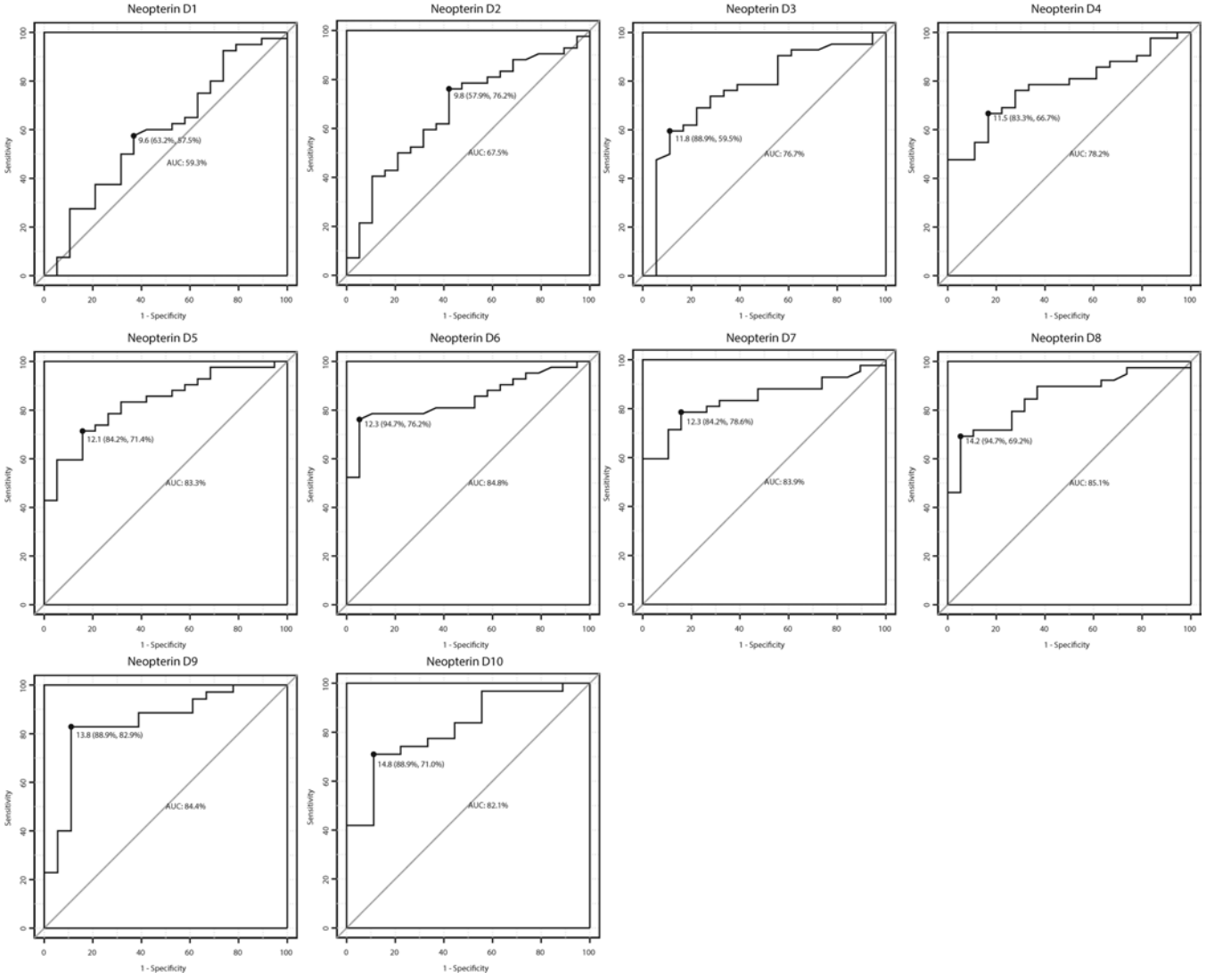

FIG. 8. ROC curves of neopterin representing the ability to differentiate between infected and uninfected patients from the admission to the hospital to 10 days after.

term condition of aSAH patients, such as infections, have not been adequately investigated.[8,10]

In our study, we focused our attention on infection because infections developed during hospitalization in 95% of the patients with poor outcomes,[10] indicating that early detection and treatment of bacterial infections may improve patient outcomes. In addition, the discrimination of infection from other pathologies with similar presentations should decrease the amount of unnecessary antibiotic use, avoiding associated resistance, toxicity, and allergic reactions.[16,33]

The WBC count is used as a reference value to detect several inflammatory diseases. However, in aSAH patients the WBC count cannot be used for this purpose because of the leukocytosis produced by the blood clot. To our knowledge, this is the first reported study showing that a marker of inflammation able to predict long-term outcome after aSAH is also correlated with the appearance of infection. Neopterin concentrations, which indicated overexpression of this biomarker in all 61 patients included in this study, enabled us to significantly differentiate between patients who developed an infection after aSAH and patients who did not. When comparing the concentrations before the detection of the infection, we found that concentrations of neopterin were already significantly different 2 days before the positive bacterial culture.

These results suggest that neopterin may be useful in clinical practice as a screening test to trigger earlier bacteriological studies. Based on our study results, the ideal day to measure neopterin concentrations seems to be 3 days after admission to the hospital; this is approximately 2 days before the detection of infection by bacterial culture. At this time point, we found that a cutoff value of 11.8 nmol/L would allow correct classification in 77% of the cases with respect to whether infection will develop or not. However, several critical issues should be further evaluated regarding the use of this biomarker. The association between infection and outcome must be clarified to confirm that prevention of infection leads to improvement in patient outcomes. In addition, the exact time or sequen-

tial times to measure the neopterin concentrations, as well as the best cutoff values to trigger initiation of treatment, must be defined.

Thus far, we have not been able to confirm that neopterin is a direct diagnostic marker of infection or whether its concentrations in aSAH patients are associated with the initial hemorrhage, which would mean that a larger hemorrhage might increase the grade of inflammation, worsening the general state of the patient and consequently increasing the risk of infection. Alternatively, further pathophysiological insights may be obtained by measuring the concentrations of neopterin in patient CSF. As already mentioned by Mathiesen et al.,[23] this polar molecule does not cross the blood-brain barrier, so the direct evaluation of the inflammation produced by the hemorrhage may increase understanding of the relation between inflammation and infection development.[23]

Furthermore, in this cohort, the development of DCI and the amounts of blood measured by the Fisher scale were not correlated with outcome, findings which do not reflect the data in the literature.[7,9,18]

In summary, once these small confounders are elucidated, neopterin could become a useful biomarker to improve the clinical management and outcome in aSAH patients.

Conclusions

Neopterin is a potential outcome and infection predictor after aSAH. The objective information provided by the measurement of neopterin concentrations immediately after hospital admission and onward may enhance the performance of other clinical methods for patient assessment. To evaluate the clinical utility of this biomarker, the results of the present study should be validated in larger and multicenter studies.

Acknowledgments

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References

1. Al-Khindi T, Macdonald RL, Schweizer TA: Cognitive and functional outcome after aneurysmal subarachnoid hemorrhage. **Stroke 41:**e519–e536, 2010
2. Beaudeux JL, Léger P, Dequen L, Gandjbakhch I, Coriat P, Foglietti MJ: Influence of hemolysis on the measurement of S-100β protein and neuron-specific enolase plasma concentrations during coronary artery bypass grafting. **Clin Chem 46:**989–990, 2000
3. Bederson JB, Connolly ES Jr, Batjer HH, Dacey RG, Dion JE, Diringer MN, et al: Guidelines for the management of aneurysmal subarachnoid hemorrhage: a statement for healthcare professionals from a special writing group of the Stroke Council, American Heart Association. **Stroke 40:**994–1025, 2009
4. Calandra T, Cohen J: The international sepsis forum consensus conference on definitions of infection in the intensive care unit. **Crit Care Med 33:**1538–1548, 2005
5. Carr KR, Zuckerman SL, Mocco J: Inflammation, cerebral vasospasm, and evolving theories of delayed cerebral ischemia. **Neurol Res Int 2013:**506584, 2013
6. Chadha S, Bhalla P, Gautam H, Chakravarti A, Saini S, Anuradha S, et al: Utility of serum neopterin and serum IL-2 receptor levels to predict absolute CD4 T lymphocyte count in HIV infected cases. **Interdiscip Perspect Infect Dis 2013:**143648, 2013
7. Chowdhury T, Dash HH, Cappellani RB, Daya J: Early brain injury and subarachnoid hemorrhage: Where are we at present? **Saudi J Anaesth 7:**187–190, 2013
8. Dhar R, Diringer MN: The burden of the systemic inflammatory response predicts vasospasm and outcome after subarachnoid hemorrhage. **Neurocrit Care 8:**404–412, 2008
9. Etminan N, Vergouwen MD, Ilodigwe D, Macdonald RL: Effect of pharmaceutical treatment on vasospasm, delayed cerebral ischemia, and clinical outcome in patients with aneurysmal subarachnoid hemorrhage: a systematic review and meta-analysis. **J Cereb Blood Flow Metab 31:**1443–1451, 2011
10. Frontera JA, Fernandez A, Schmidt JM, Claassen J, Wartenberg KE, Badjatia N, et al: Impact of nosocomial infectious complications after subarachnoid hemorrhage. **Neurosurgery 62:**80–87, 2008
11. Fujii M, Yan J, Rolland WB, Soejima Y, Caner B, Zhang JH: Early brain injury, an evolving frontier in subarachnoid hemorrhage research. **Transl Stroke Res 4:**432–446, 2013
12. Haug T, Sorteberg A, Sorteberg W, Lindegaard KF, Lundar T, Finset A: Cognitive outcome after aneurysmal subarachnoid hemorrhage: time course of recovery and relationship to clinical, radiological, and management parameters. **Neurosurgery 60:**649–657, 2007
13. Hong CM, Tosun C, Kurland DB, Gerzanich V, Schreibman D, Simard JM: Biomarkers as outcome predictors in subarachnoid hemorrhage—a systematic review. **Biomarkers 19:**95–108, 2014
14. Iosif C, Di Maria F, Sourour N, Degos V, Bonneville F, Biondi A, et al: Is a high initial World Federation of Neurosurgery (WFNS) grade really associated with a poor clinical outcome in elderly patients with ruptured intracranial aneurysms treated with coiling? **J Neurointerv Surg 6:**286–290, 2014
15. Kimball MM, Velat GJ, Hoh BL: Critical care guidelines on the endovascular management of cerebral vasospasm. **Neurocrit Care 15:**336–341, 2011
16. Korinek AM, Baugnon T, Golmard JL, van Effenterre R, Coriat P, Puybasset L: Risk factors for adult nosocomial meningitis after craniotomy: role of antibiotic prophylaxis. **Neurosurgery 62 (Suppl 2):**532–539, 2008
17. Lagares A, Gómez PA, Alen JF, Lobato RD, Rivas JJ, Alday R, et al: A comparison of different grading scales for predicting outcome after subarachnoid haemorrhage. **Acta Neurochir (Wien) 147:**5–16, 2005
18. Latorre JG, Lodi Y, El-Zammar Z, Devasenapathy A: Is asymptomatic vasospasm associated with poor outcome in subarachnoid hemorrhage? **Neurohospitalist 1:**165–171, 2011
19. Lhee HY, Kim H, Joo KJ, Jung SS, Lee KB: The clinical significance of serum and urinary neopterin levels in several renal diseases. **J Korean Med Sci 21:**678–682, 2006
20. Li ZQ, Wang QH, Chen G, Quan Z: Outcomes of endovascular coiling versus surgical clipping in the treatment of ruptured intracranial aneurysms. **J Int Med Res 40:**2145–2151, 2012
21. Linn FHH, Rinkel GJE, Algra A, van Gijn J: Incidence of subarachnoid hemorrhage: role of region, year, and rate of computed tomography: a meta-analysis. **Stroke 27:**625–629, 1996
22. Maddahi A, Povlsen GK, Edvinsson L: Regulation of enhanced cerebrovascular expression of proinflammatory mediators in experimental subarachnoid hemorrhage via the

mitogen-activated protein kinase kinase/extracellular signal-regulated kinase pathway. **J Neuroinflammation 9:**274, 2012
23. Mathiesen T, Fuchs D, Wachter H, von Holst H: Increased CSF neopterin levels in subarachnoid hemorrhage. **J Neurosurg 73:**69–71, 1990
24. McMahon CJ, Hopkins S, Vail A, King AT, Smith D, Illingworth KJ, et al: Inflammation as a predictor for delayed cerebral ischemia after aneurysmal subarachnoid haemorrhage. **J Neurointerv Surg 5:**512–517, 2013
25. Molyneux AJ, Kerr RS, Yu LM, Clarke M, Sneade M, Yarnold JA, et al: International subarachnoid aneurysm trial (ISAT) of neurosurgical clipping versus endovascular coiling in 2143 patients with ruptured intracranial aneurysms: a randomised comparison of effects on survival, dependency, seizures, rebleeding, subgroups, and aneurysm occlusion. **Lancet 366:**809–817, 2005
26. Murr C, Widner B, Wirleitner B, Fuchs D: Neopterin as a marker for immune system activation. **Curr Drug Metab 3:**175–187, 2002
27. Oertel M, Schumacher U, McArthur DL, Kästner S, Böker DK: S-100B and NSE: markers of initial impact of subarachnoid haemorrhage and their relation to vasospasm and outcome. **J Clin Neurosci 13:**834–840, 2006
28. Oshiro EM, Walter KA, Piantadosi S, Witham TF, Tamargo RJ: A new subarachnoid hemorrhage grading system based on the Glasgow Coma Scale: a comparison with the Hunt and Hess and World Federation of Neurological Surgeons Scales in a clinical series. **Neurosurgery 41:**140–148, 1997
29. Provencio JJ: Inflammation in subarachnoid hemorrhage and delayed deterioration associated with vasospasm: a review. **Acta Neurochir Suppl 115:**233–238, 2013
30. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al: pROC: an open-source package for R and S+ to analyze and compare ROC curves. **BMC Bioinformatics 12:**77, 2011
31. Sanchez-Peña P, Pereira AR, Sourour NA, Biondi A, Lejean L, Colonne C, et al: S100B as an additional prognostic marker in subarachnoid aneurysmal hemorrhage. **Crit Care Med 36:**2267–2273, 2008
32. Schievink WI: Intracranial aneurysms. **N Engl J Med 336:**28–40, 1997
33. Simon L, Gauvin F, Amre DK, Saint-Louis P, Lacroix J: Serum procalcitonin and C-reactive protein levels as markers of bacterial infection: a systematic review and meta-analysis. **Clin Infect Dis 39:**206–217, 2004
34. Suarez JI, Tarr RW, Selman WR: Aneurysmal subarachnoid hemorrhage. **N Engl J Med 354:**387–396, 2006
35. Tiberti N, Lejon V, Hainard A, Courtioux B, Robin X, Turck N, et al: Neopterin is a cerebrospinal fluid marker for treatment outcome evaluation in patients affected by Trypanosoma brucei gambiense sleeping sickness. **PLoS Negl Trop Dis 7:**e2088, 2013
36. Turck N, Vutskits L, Sanchez-Pena P, Robin X, Hainard A, Gex-Fabry M, et al: A multiparameter panel method for outcome prediction following aneurysmal subarachnoid hemorrhage. **Intensive Care Med 36:**107–115, 2010
37. Wang HC, Lin WC, Yang TM, Lin YJ, Tsai NW, Cheng KY, et al: The association between symptomatic delayed cerebral infarction and serum adhesion molecules in aneurysmal subarachnoid hemorrhage. **Neurosurgery 68:**1611–1617, 2011
38. Weiss N, Sanchez-Peña P, Roche S, Beaudeux JL, Colonne C, Coriat P, et al: Prognosis value of plasma S100B protein levels after subarachnoid aneurysmal hemorrhage. **Anesthesiology 104:**658–666, 2006
39. Yoshimoto Y, Tanaka Y, Hoya K: Acute systemic inflammatory response syndrome in subarachnoid hemorrhage. **Stroke 32:**1989–1993, 2001

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Sanchez, Azurmendi, Tiberti. Acquisition of data: Azurmendi. Analysis and interpretation of data: Azurmendi, Tiberti, Kapandji, Turck. Drafting the article: Sanchez, Azurmendi, Tiberti. Critically revising the article: Sanchez, Degos, Tiberti. Reviewed submitted version of manuscript: Sanchez, Turck. Approved the final version of the manuscript on behalf of all authors: Sanchez. Statistical analysis: Azurmendi, Degos, Tiberti, Turck. Study supervision: Sanchez, Sanchez-Peña, Sarrafzadeh, Puybasset, Turck.

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Chapter IV

Measuring serum amyloid-A for infection prediction in aneurysmal subarachnoid hemorrhage

Measuring serum amyloid-A for infection prediction in aneurysmal subarachnoid hemorrhage

Leire Azurmendi, Vincent Degos, Natalia Tiberti, Natacha Kapandji, Paola Sanchez, Asita Sarrafzadeh, Louis Puybasset, Natacha Turck, Jean-Charles Sanchez

As already described in the previous chapter, infections occurring after aSAH have deleterious effects on patient's outcome. The prompt identification of patients at high risk of developing them, could allow an earlier antibiotherapy and improvement on patients outcome. Therefore, the main objective of this chapter was to find an infection biomarker earlier than neopterin in order to start with the treatment as soon as the diagnosis has been performed. For this purpose, we compared the proteome of infected and non-infected patients at hospital admission using a TMT-6 plex. Among the 209 quantified proteins and among the 17 significantly regulated ones, SAA appeared to be the protein with the most promising ratio, being 15.86 (SD: 5.1-49.3) times more elevated in infected patients than in non-infected ones. This discovery result was validated using ELISA immunoassays in 81 patients and confirmed that SAA could be a promising infection marker in aSAH.

The corresponding article was published in 2015 in *Journal of proteome research.* My contribution consisted in performing the different experiments, analyzing the data and writing the article.

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Measuring Serum Amyloid A for Infection Prediction in Aneurysmal Subarachnoid Hemorrhage

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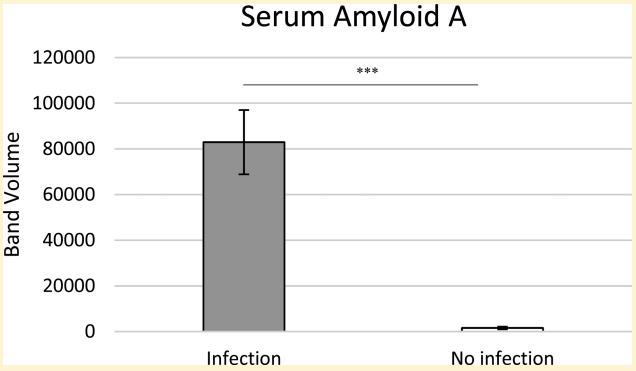
Supporting Information

ABSTRACT: Aneurysmal subarachnoid hemorrhage (aSAH) is associated with high rates of mortality and morbidity. Nosocomial infections, such as pneumonia or urinary tract infections, are among the main causes of worsening outcomes and death. The aim of this study was to discover a biomarker to predict infection in aSAH patients. For this purpose, the plasma of infected and noninfected patients was compared using quantitative mass spectrometry. The most interesting differentially expressed proteins were selected for validation by immunoassays on plasma samples taken from patients (n = 81) over 10 days of hospitalization. Predictive performances were established using Mann−Whitney U tests and receiver operating characteristic curves. Quantitative proteomics identified 17 significantly regulated proteins. Of these, levels of serum amyloid A (SAA) were significantly higher in infected patients (p < 0.007). ELISA confirmed that the concentrations were significantly higher (p < 0.002) already at hospital admission in patients who subsequently developed an infection during their hospitalization, (AUC of 76%) for a cutoff value of 90.9 μg/mL. Our data suggested that measuring SAA could be an efficient means of detecting patients susceptible of developing an infection during hospitalization after an aSAH. Its predictive capacity could lead to earlier antibiotherapy, improved patient management, and potentially better long-term outcomes.



KEYWORDS: *collision-induced dissociation, nosocomial infection, serum amyloid A, Glasgow Coma Scale, Glasgow Outcome Scale*

1. INTRODUCTION

aSAH is a devastating condition produced by the rupture of a cerebral aneurysm.[1] Approximately 35% of the patients die within 24 h of the initial hemorrhage and many survivors remain hospitalized with important physical and neurological impairments.[2−4]

The impact of the initial hemorrhage produces a local and general inflammatory response that affects patients' long-term states of health.[5] aSAH increases the levels of inflammatory factors, and a high number of lymphocytes are released into the brain.[6] Significant immunodepression is subsequently observed, producing an impairment of immune function. A reduction in the number of T and B cells is observed, as is a decrease in the activation of those T cells.[7,8]

This imbalance in the immune system increases the risk factors for developing secondary complications such as infection, which is both common and significant in aSAH patients.[8] Twenty percent of hospitalized patients develop pneumonia, 13% develop a urinary tract infection, 8% develop a bloodstream infection, and 5% develop meningitis or ventriculitis.[9]

In stroke patients, these infections have been described as being significant outcome modulators, prolonging hospital stays and increasing rates of morbidity and mortality.[10−12] Very little is known about whether and how infections in aSAH patients affect their long-term health status; however, it has been postulated that the early diagnosis and prevention of infections could lead to better management of aSAH patients.[9]

Symptoms that usually indicate the presence of an infection are importantly unsettled by the initial hemorrhage. Concerning biomarker discovery, we recently showed that the levels of neopterin—a catabolic product of guanosin-5-triphosphate (GTP)—correlated with the presence of infection in aSAH patients.[13] Other studies have also investigated the capacity for procalcitonin and C-reactive protein (CRP) to be sepsis markers in aSAH patients.[14] Currently, however, an ideal

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biomarker with potential to be translated into the clinical practice has yet to be found.

The aim of the present study was to find an early biomarker that could identify aSAH patients who are at a higher risk of developing infectious complications, to change clinical practices, and to improve their long-term health status.[15] Using proteomics, we compared the plasma samples of infected and noninfected aSAH patients to identify the most promising biomarkers. Because SAA levels were already found to be high at hospital admission, this made it a promising infection detection tool.

2. MATERIAL AND METHODS

2.a. Sample and Patient Descriptions

The 81 aSAH patients included in this study were hospitalized at the Pitié Sapêtrière hospital in Paris (France) between July 2004 and April 2008. The present study was approved by the hospital's ethics committee (Comité de Protection des Personnes, Pitié Sapêtrière, Paris, France). All patients or their legal representatives signed an informed consent form.

Patients fulfilled the inclusion criteria for enrollment if their aSAH event had been confirmed by angiograph and computed tomography, they were admitted to hospital within 2 days of the initial hemorrhage, and they were aged above 18 years old. Exclusion criteria were any missing clinical information, not enough sample volume, and more than one hemorrhagic event.

At hospital admission clinical scores were used to assess patients' severity. The World Federation of Neurosurgical Societies scale (WFNS 1−5) was used to dichotomize the patients in good (WFNS 1−2) and poor (WFNS 3−5) clinical state. The Glasgow Coma Scale (GCS 3−15) was used to determinate the grade of the brain injury: patients with severe brain injury (GCS < 9), with moderate brain injury (GCS 9−12) and with minor brain injury (GCS ≥ 13).[16,17] The severity of the hemorrhage was determined using the Fisher score.[18]

Patients' clinical outcomes were established by telephone interview 1 year after the hemorrhage, using the Glasgow Outcome Scale (GOS). Patients were classified as having either a poor (GOS score of 1, 2, or 3) or good outcome (GOS score of 4 or 5) depending on their level of functional dependence.[19]

Plasma samples were collected from the patients every morning, from hospital admission until 10 days later (D1−D10). Infection status was established according to the International Sepsis Forum Consensus Conference on Definitions of Infection in the ICU.[20] Antibiotherapy was only started when systematic clinical and biological criteria pointed to a bacterial infection such as pneumonia, a urinary tract infection, or a bloodstream infection. Bacteriological samples were taken at this time point and the treatment was adjusted once the results were obtained.

2.b. Proteomic Study

Quantitative proteomic analyses were performed on three infected patients, 2 days before the infection developed, and on two noninfected patients to identify any differentially expressed proteins.

2.b.i. Sample Preparation. Depletion with Resin. Ten μL of each plasma sample was depleted of the 12 most abundant proteins (alpha-1-acid glycoprotein, alpha-1-antitrypsin, alpha-2-macroglobulin, albumin, apolipoprotein A-I, apolipoprotein A-II, fibrinogen, haptoglobin, IgA, IgG, IgM, transferrin) using Proteome Purify 12 immunodepletion resin (R&D Systems), according to the manufacturer's instructions. Protein concentrations were determined using the Bradford assay. Two μg of protein from each sample was analyzed using the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedure to evaluate the proper depletion performance of the 12 abundance proteins. Samples were dried in a speed vacuum.

2.b.ii. Reduction, Alkylation, Digestion, and TMT Labeling. The quantitative proteomic experiment used 25 μg of protein from each depleted sample. These dried samples were added to 33 μL of tetraethylammonium bromide (TEAB) to reconstitute them. The proteins were reduced by adding 2 μL of tris(2-carboxyethil) phosphine hydrochloride (TCEP) to each sample; they were then reacted at 37 °C for 60 min. This solution was mixed with iodoacetamide 400 mM for the alkylation step, and this mixture was stored in the dark for 30 min at room temperature. The digestion occurred overnight at 37 °C using trypsin (0.2 μg/μL). The protocol is detailed by Dayon et al.[21,22]

Subsequently, 25 μg of each digested plasma sample was labeled with one of the six TMT reagents (Proteome Sciences, Frankfurt, Germany) applying a TMT-based approach, that is, Simultaneous Marker discovery And verification for the Rapid Translation of Exogenous Reference material (SMARTER) and according to the manufacturer's instructions. Samples belonging to infected patients were labeled with TMTs 126, 127, and 128. Samples belonging to noninfected patients were labeled with TMTs 129 and 130. TMT 131 was used to label a mixture of 25 μg of white blood cells (WBCs) and 25 μg of postmortem cerebrospinal fluid (CSF). Each sample was spiked with 1 μg of β-lactoglobulin to monitor experimental error. The total quantity of each labeled sample (25 μg) was pooled and dried in a speed-vacuum.

2.b.iii. Off-Gel Electrophoresis. Off-gel electrophoresis (OGE) separation was carried out using an Agilent 3100 Off-Gel fractionator, according to the manufacturer's instructions. Previously dried samples were reconstituted using the OGE solution and focused using an immobilized pH gradient (IPG) dry strip (13 cm, pH 3−10).[21] Samples were desalted, dried in the speed vacuum, and stored at −20 °C until analysis.

2.b.iv. LC−MS/MS. An LTQ Orbitrap Velos Pro instrument (Thermo, San Jose, CA) coupled to a nanoflow high-pressure liquid chromatography (NanoAquity system (Waters)) was used to analyze the OGE fractions, as previously described.[23]

In brief, peptides were trapped in a homemade 5 μm 200 Å Magic C18 AQ (Michrom) 0.1 × 20 mm precolumn and separated on a Technology analytical nanocolumn (C18, 5 μm, 100 Å). The analytical separation was run for 65 min using a gradient of H_2O/FA 99.9%/0.1% (solvent A) and CH_3CN/FA 99.9%/0.1% (solvent B). The gradient was run as follows: 0−1 min 95% A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min at a flow rate of 220 nL/min. For the MS survey scans, OT resolution was set to 60 000 and the ion population was set to 5×10^5 with an m/z window from 400 to 2000. A maximum of three precursors were selected for both collision-induced dissociation (CID) in the LTQ and high-energy C-trap dissociation (HCD) with analysis in the OT. For MS/MS in the LTQ, the ion population was set to 7×10^3 (isolation width of 2 m/z), while for MS/MS detection in the OT it was set to 2×10^5 (isolation width of 2.5 m/z), with a resolution of 7500, first mass at m/z = 100, and maximum injection time of 750 ms. The normalized collisional energies were set to 35% for CID and 60% for HCD. The dynamic

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Table 1. Demographic Characteristics of the Study Population Comparing the Presence or Absence of Infection during Hospitalization

		Infection		
	Total n = 81 (%)	Yes (n = 54)	No (n = 27)	P^a
Gender				0.099
Male n (%)	28 (34.6%)	22 (27.2%)	6 (7.4%)	
Female n (%)	53 (65.4%)	32 (39.5%)	21 (25.9%)	
Age (years)[b]				0.455
Average (±SD)	51.3 ± 13	52.1 ± 12.87	49.81 ± 13.43	
WFNS score				**0.001**
1,2 n (%)	48 (59.3%)	25 (30.9%)	23 (28.4%)	
3,4,5 n (%)	33 (40.7%)	29 (35.8%)	4 (4.9%)	
Glasgow Coma Scale				**0.001**
<9	21 (25.9%)	20 (24.7%)	1 (1.2%)	
9−12	8 (9.9%)	7 (8.6%)	1 (1.2%)	
≥13	52 (74.1%)	27 (33.3%)	25 (30.9%)	
Vasospasm				0.111
Yes n (%)	34 (42%)	26 (32.1%)	8 (9.9%)	
No n (%)	47 (58%)	28 (34.6%)	19 (23.5%)	
Treatment				0.276
Surgery n (%)	63 (77.8%)	42 (51.9%)	21 (25.9%)	
Embolization n (%)	14 (17.3%)	8 (9.9%)	6 (7.4%)	
No treatment n (%)	4 (4.9%)	4 (4.9%)		
EVD				**<0.0001**
Yes n (%)	52 (64.2%)	44 (54.3%)	8 (9.9%)	
No n (%)	29 (35.8%)	10 (12.3%)	19 (23.5%)	
GOS 1 year[c]				**0.002**
Good n (%)	45 (55.6%)	26 (32.1%)	19 (23.5%)	
Poor n (%)	21 (44.4%)	20 (24.7%)	1 (1.2%)	

[a]Fisher's exact test/Chi square test. [b]Age: Mann−Whitney U test. [c]GOS score 1, 2, or 3: poor outcome; GOS score 4 or 5: good outcome.

exclusion was fixed to 45 s. The charged-state screening parameters were set to exclude precursor ions of charge state 1+ for MS analysis.

2.b.v. Data Analysis. *Protein Identification.* Peak lists were generated from the combined HCD-CID raw data spectra using EasyProtConv v1.5 software. The peak lists were obtained using the 12 OGE fractions and were submitted to the EasyProt software platform (version 2.3, build 718) that uses the Phenyx software (GeneBio, Geneva, Switzerland) for protein identification. Searches were performed using the Uniprot-Swiss Prot database (2014-10, 669903).[24] A *Homo sapiens* taxonomy was selected to search this database, and the following parameters were selected: Oxidized methionine was set as the variable modification and cysteine carbamethylation, and TMT^6 lysine and TMT^6 amino-terminus were set as the fixed modifications. Trypsin was selected as the proteolytic enzyme, allowing one missed cleavage. The parent ion tolerance was set to 10 ppm, and the accuracy of fragment ions was set to 0.6 Da. Only proteins with <1% false discovery rate (FDR) and at least two different unique peptides were selected for further analysis.[25] A minimum peptide length of six amino acids was used.

Protein Quantification. Isobaric quantification was performed using the Isobar R package.[26] Isotopic impurities of TMT^6 reporter-ion intensities were corrected according to the isotopic distribution data provided by the manufacturer. Intensities were also normalized by using the equal median intensity method. Peptides were not quantified without reporter intensities. The infection/no infection ratio was calculated for each peptide, combining the reporter-ion intensities between infected patient channels (126.1, 127.1, and 128.1) and noninfected patient channels (129.1 and 130.1). To test the ratio's accuracy and biological significance, we calculated technical and biological variability for each protein ratio. A ratio p value and a sample p value were calculated for each variable. Furthermore, only proteins with a cutoff threshold value higher or lower than 1.5 or 0.67 were considered.[27−29]

2.c. Western Blotting

Western blotting (WB) was used to evaluate the expression of serum amyloid A (SAA) taken from six infected patients (2 days before infection onset) and four noninfected patients at the equivalent time point. For each patient, 3 μL of 1/100 diluted plasma was loaded onto a 15% T/2.6% C acrylamide gel. Mouse anti-SAA monoclonal antibody (Abcam, Cambridge, U.K.) was used at a dilution of 1/4000. The secondary antibody (polyclonal goat antimouse horseradish peroxidase, Dakon Denmark, Glostrup, Denmark) was applied at 1/2000 dilution. The images obtained were analyzed using ImageQuant 7.0 software (GE Healthcare). SAA band intensities from infected and noninfected patients were then analyzed using GraphPad Prism software (version 6.03, 2013, San Diego, CA).

2.d. ELISA

To determine levels of SAA from hospital admission to 10 days later, plasma samples were diluted 1/2000. The Meso Scale Discovery (MSD) Vascular Injury Panel-I ECL assay was used according to the manufacturer's instructions (MSD, Gaithersburg, MD). SAA concentrations were assessed using an electrochemiluminescence detection system using multiarray technology (SECTOR Imager 2400, Meso Scale Discovery), as previously described.[30]

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Table 2. Significant Differential Plasma Proteins between Infected and Noninfected Patients after Proteomic Analysis

Protein		ID	AC	Peptides	Ratio	p value rat	p value sample
Peroxiredoxin-1	PRDX1	PRDX1_HUMAN	Q06830	2	0.3	<0.0001	0.005
Histidine-rich glycoprotein	HRG	HRG_HUMAN	P04196	10	0.54	<0.0001	0.008
Lumican	LUM	LUM_HUMAN	P51884	7	0.58	<0.0001	0.010
Antithrombin-III	SERPINC	ANT3_HUMAN	P01008	18	0.63	<0.0001	0.016
Gelsolin	GSN	GELS_HUMAN	P06396	12	0.65	<0.0001	0.019
Tetranectin	CLEC3B	TETN_HUMAN	P05452	5	0.67	0.037	0.040
Kallistatin	SERPINA4	KAIN_HUMAN	P29622	9	0.74	0.010	0.043
SH3 domain binding glutamic acid rich like protein 3	SH3BGRL3	SH3L3_HUMAN	Q9H299	2	1.39	0.011	0.049
Carbonic anhydrase 2	CA2	CAH2_HUMAN	P00918	3	1.48	<0.0001	0.026
Complement factor H-related protein 4	CFHR4	FHR4_HUMAN	Q92496	2	1.54	0.044	0.028
Von Willebrand factor	VWF	VWF_HUMAN	P04275	5	1.7	0.013	0.009
Alpha-1-acid glycoprotein 1	ORM1	A1AG1_HUMAN	P02763	8	1.74	0.047	0.006
Neutrophil defensin 3	DEFA3	DEF3_HUMAN	P59666	6	2.02	0.000	0.004
Haptoglobin	HP	HPT_HUMAN	P00738	26	2.1	0.001	0.001
Pregnancy zone protein	PZP	PZP_HUMAN	P20742	10	2.55	0.010	0.004
Hemoglobin subunit beta	HBB	HBB_HUMAN	P68871	8	3.7	0.021	0.002
Serum amyloid A-1 protein	SAA1	SAA1_HUMAN	P0DJI8	8	15.86	0.007	<0.0001

2.e. Statistical Analysis

We used SPSS software (version 21, SPSS, Chicago, IL) for the statistical analyses. Because the levels of SAA did not follow a normal distribution, the Mann−Whitney U test was used for statistical comparisons between two unpaired groups. A Bonferroni correction of the p values was applied when multiple comparisons were performed.

All statistical tests were bilateral, and a p value <0.05 was considered statistically significant.

Receiver operating characteristic (ROC) curves were calculated for SAA at hospital admission. The values of area under the ROC curves (AUC), specificity (SP), sensitivity (SE), and 95% confidence intervals (95% CI) were calculated using the pROC package for S+, version 8.1. (TIBCO, Software Inc.).[31]

Univariate and multivariate analyses were performed to assess the associations between variables. The presence of infection was set as the dependent variable, and the WFNS score, GCS score, SAA, and an external ventricular drain (EVD) were set as confounders. Categorical data were dichotomized according to the criteria from the table of demographic characteristics. Longitudinal data were also dichotomized according to the best cutoff points obtained after analysis from the AUC values.

3. RESULTS

3.a. Population

The present study included 81 aSAH patients: 27 noninfected and 54 infected. Of the infected patients, 83% developed pneumonia and the remaining 17% developed a urinary tract infection. Their demographic characteristics are shown in Table 1. The mean patient age was 51.3 years old ±13; 65.4% of patients were women; 59.3% of patients were in a good neurological state (WFNS score 1−2) at hospital admission; 42% of patients developed a vasospasm (confirmed by angiograph)during hospitalization; and 64.2% had an EVD installed. At 1 year, 44.4% of patients were rated as having a poor health outcome (GOS 1, 2, or 3).

The WFNS and GCS scores were highly associated with the development of an infection: Patients with a poor neurological state at hospital admission were more prone to developing an infection than those under good neurological condition (p = 0.001). Patients with an EVD were also at a higher risk of developing an infection (p < 0.0001). Finally, we observed that 95% of the patients with a poor health outcome at 1 year had developed an infection.

3.b. Proteomic Workflow

3.b.i. Discovery Step: Proteomic Results. Quantitative proteomic analyses comparing the proteome of infected and noninfected patients revealed 209 proteins (1% FDR, two unique peptides) (Supplementary Table 1). Seventeen proteins were significantly regulated between the two groups: 7 down-regulated and 10 up-regulated proteins were found in patients who went on to develop an infection during their stay in hospital (Table 2). The most significantly expressed of the 17 proteins (serum amyloid A, with a ratio of 15.86 (SD: 5.1−49.3) to 1) was selected for further analysis.

3.b.ii. Verification Step: WB Results. To verify the SAA results obtained via the proteomic TMT^6 analysis, we made a WB analysis of the plasma samples from six infected aSAH patients (2 days before the infection developed) and four noninfected aSAH patients. Half of the patients included in this verification phase had been included in the proteomic discovery phase and half were new patients. As shown in Figure 1, the intensity of plasma bands for all of the infected aSAH patients

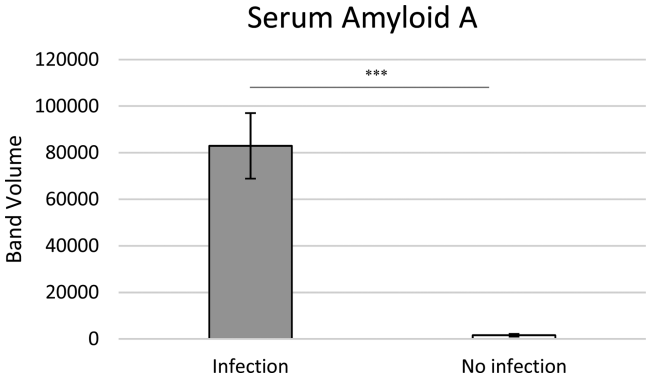

Figure 1. Western blotting expression of SAA in plasma samples of 6 infected patients (2 days before the infection developed) and 4 noninfected patients. * = significance level (p < 0.05).

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was significantly higher than those for noninfected ones ($p < 0.001$), confirming the results obtained using quantitative proteomics.

3.b.iii. Validation Step: ELISA Results. SAA levels in the plasma of 54 infected and 27 noninfected patients were measured using an SAA ELISA assay. Concentrations were measured daily, from hospital admission until 10 days later. Figure 2 shows that throughout hospitalization the mean SAA levels were significantly higher in infected patients than in noninfected ones.

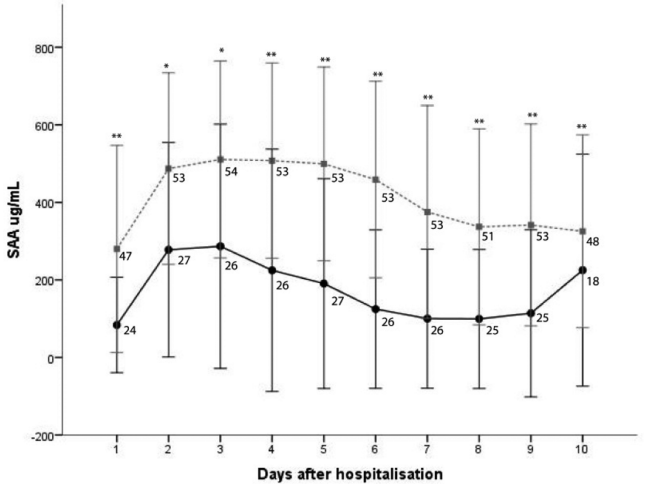

Figure 2. Kinetics of SAA concentrations from hospital admission through to 10 days later. The mean SAA concentration is shown by ■ for infected patients and by ● for noninfected patients. Bars represent the standard deviation to the mean. The numbers on the graphs represent the number of patients tested at each time point. Comparisons between the two groups were made using the Mann–Whitney U test. * = significance level reported after the Bonferroni correction ($p < 0.05$).

The accuracy of using SAA to differentiate between these two groups of patients was evaluated at hospital admission (D1) and at the mean day of infection development (D5). As shown in Figure 3, 5 days after hospitalization, using SAA to discriminate between patients who would develop an infection and those who would not, had reached a total AUC value of 79.4% (cutoff: 91.2 μg/mL). Furthermore, we found that even at hospital admission, this method of differentiation had already reached a promising AUC value of 76% (cutoff: 90.96 μg/mL).

To better understand its capacity to determine which patients would develop an infection, we fixed the SP value of 100%, which gave a SE value of 25% (threshold: 514.1 μg/mL). This signified that at hospital admission, 100% of the patients who would not develop an infection could be correctly classified, as could 25% of the patients who would.

3.c. Univariate and Multivariate Analysis

Taking into account the cutoff concentration obtained at hospital admission to discriminate between infected and noninfected patients (threshold: 90.96 μg/mL), we dichotomized the SAA continuous variable to perform a regression analysis.

Univariate analyses showed that the WFNS score, the GCS score, SAA, and EVD were all associated with the presence of infection in aSAH patients; however, in multivariate analyses, only SAA levels and the presence of an EVD showed a relationship with the development of an infection. Patients with SAA levels higher than 90.96 μg/mL on admission had a higher risk (5.8 times higher) of developing an infection than patients with values below this cutoff (Table 3).

4. DISCUSSION

Infections occurring in the first few days following an aSAH are associated with important rates of morbidity and mortality; therefore, this study aimed to identify infection predictor markers to reduce the number of infections occurring after hemorrhagic events. Using proteomics, we compared plasma samples from infected and noninfected aSAH patients to identify the most promising biomarkers. Among the significantly regulated proteins between the two groups, SAA showed the most promising ratio. The elevated concentrations of SAA

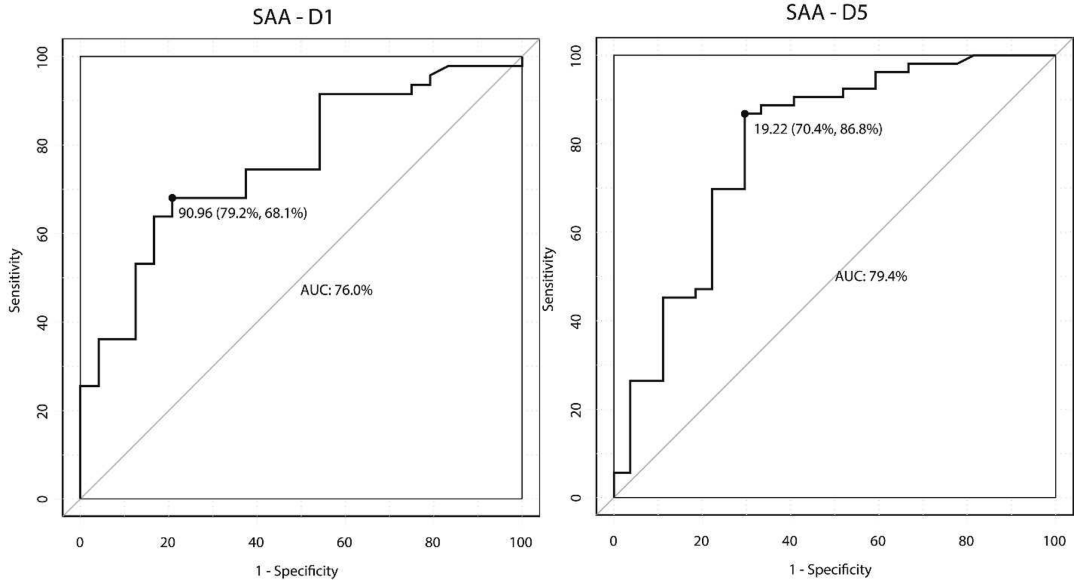

Figure 3. ROC curve of SAA representing the capacity to differentiate between patients who would and would not develop an infection during their hospitalization: at hospital admission (D1) and at the mean day of infection (D5).

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Table 3. Univariate and Multivariate Analyses of Different Parameters for Predicting the Presence of Infection at Hospital Admission[a]

Presence of infection	*p*	OR
Univariate Analysis		
WFNS score	0.002	6.67 (2.03−21.9)
GCS score		
13−15	reference	
<9	0.006	18.51(2.31−148.34)
SAA at admission (90.9 ug/mL)	**<0.0001**	8.11 (2.54−25.87)
EVD	**<0.0001**	10.45(3.57−30.59)
Multivariate Analysis		
SAA at admission (90.9 μg/mL)	**0.01**	5.82 (1.52−22.31)
EVD	**0.04**	4.03(1.08−15.07)

[a]Table details *p* values and the odds ratio (OD) with 95% CI. The dichotomization of longitudinal data was made at the cut-off obtained in the AUC analysis at D1. The values applied are written next to the variable.

found in infected patients through proteomic analysis were confirmed using WB and ELISA, making SAA levels a promising diagnostic tool for infection.

At present, infection diagnosis is a complex task for physicians: Symptoms that usually indicate the presence of an infection, such as fever or high levels WBC, are merely indicators of an aSAH patient's initial brain injury, as produced by the hemorrhage.[32] When infection is suspected, therefore, a bacterial culture is made, but a result can take 2 or 3 days.

A more objective parameter with which to identify the patients at a higher risk of infection could improve antibiotherapy, patient management, and, consequently, the associated outcome.[10,33]

Copeptin levels appear to be higher in stroke patients who develop an infection than in control patients.[34] The use of procalcitonin as a diagnostic tool for general and respiratory tract infections associated with strokes has also been previously studied;[35,36] however, the associated low SE and controversies between studies have brought into question procalcitonin's utility in clinical practice.

To the best of our knowledge, there has been little research on infection diagnosis in aSAH patients. Neopterin has been one of the most promising markers to date.[13] This biomarker of inflammation correlated well with infection. Levels of neopterin were significantly higher in infected patients than in non-infected ones at 4 days after hospitalization and gave an AUC value of 74.8% at that time point. Nevertheless, an ideal biomarker would already be able to correctly classify which patients will develop an infection at hospital admission, and thus this should occur earlier than with neopterin.

We postulated that the development of a systemic infection would trigger important changes in the proteins circulating in plasma some days before the onset of that infection. We applied quantitative proteomics to identify biomarkers detectable earlier than neopterin.

Among the 17 differentially regulated proteins at admission to the hospital, some of them were already described as being correlated with infection. Concentrations of neutrophil defensin 3, for example, appeared to be elevated in plasma, blood, and body fluids from patients with infection.[37] Haptoglobin and ORM-1, two acute phase proteins, also showed increased levels in inflammatory and infectious disorders;[38−40] however, in this study, SAA showed the most promising elevated ratio. It is an acute phase inflammatory protein produced by the liver and the precursor of the amyloid A protein.[41,42] Increased concentrations of SAA have been found in patients suffering from malignant tumors, autoimmune diseases, and viral (influenza, rhinovirus, rubella) and bacterial (enterocolitis, urinary tract infection) infections.[43−45] It has been also described as an important diagnostic marker of neonatal sepsis.[46,47] In cerebral infarction, different concentrations of SAA were found in patients with and without infectious complications.[48]

This study, validated on 81 patients, showed that SAA levels at hospital admission were significantly higher in patients who went on to develop an infection during their hospitalization than in patients who did not. SAA concentrations start increasing in infected patients just after their aSAH and remained elevated throughout their hospitalization, which is in perfect agreement with previously described SAA kinetics.[47] Compared with previous studies of neopterin, we observed that SAA was more accurate and could predict infection earlier, making this biomarker a promising diagnostic tool for infection.

The increase obtained at the admission to the hospital could be considered to be a marker of vulnerability in aSAH patients, being an indirect indicator of infection. Consequently, testing SAA levels could be used clinically to flag patients who should be monitored more closely.[49] Physicians could modify the management of higher risk patients right from admission. This could help to avoid invasive care/treatment, to increase preventive measures to avoid pulmonary infections and, most importantly, to monitor clinical and laboratory indicators of infection to start antibiotherapy as soon as the infection appears.[34,50,51] The moment when antibiotherapy should begin has been extensively studied and remains a controversial subject with regard to critical care patients. Some phase II clinical trials have shown that the administration of prophylactic therapy could reduce the number of infections and in some cases improve the patient outcomes;[52−54] however, other studies demonstrated that the intravenous administration of levofloxacin did not improve infection prevention when compared with optimal care.[55]

Our study stands with several technical and biological limitations.

Technically, the discovery TMT approach used to compare the proteome of infected and noninfected patient was performed in a very small sample size (n = 5), which could lead to several false-positive and -negative results. When it is applied for discovery steps, this drawback should always be considered and be by strict verification and validation steps. Biologically, for SAA to become an effective biomarker of infection, leading to prophylactic treatment, the present study's results should be validated in a prospective multicenter and larger cohort; the number of patients included in this study was limited, especially when the subgroup of patients (infection/noninfection) was stratified. Furthermore, it should be investigated whether SAA can act as a diagnostic marker of infection or whether it is a marker of inflammation. The differential diagnosis between inflammation and infection is crucial to avoid unnecessary antibacterial treatment. To do this, the levels of SAA should be compared for systemic bacterial infections, viral infections, and inflammatory events without infectious complications.[35] Similarly, the lack of SP in distinguishing the different types of infection is an important drawback of acute-phase inflammatory molecules that should be solved. As previously reported, the combination of SAA with

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other markers or clinical parameters could lead to promising results. Finally, an explanation of SAA's role in aSAH should be sought. The results presented here, together with those from neopterin studies, suggest that inflammation has an important role in the pathophysiology of the disease; however, there is still a need for an explanation of why SAA levels increased before clinical signs of infection appeared.[56]

Once these confounders have been elucidated, SAA could be added the list of biological parameter used in clinical practice to improve the management of patients at risk of infection and associated outcomes.

5. CONCLUSIONS

In a small cohort of patients with aSAH, the present study demonstrated that SAA levels could be used to predict infection already at hospital admission. This suggests that SAA is a promising tool for future use in clinical practice to improve the management of aSAH patients. Further prospective, multicenter studies are needed to validate these results.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00391.

Quantitative proteomic analyses comparing the proteome of infected and noninfected patients revealed 209 proteins (1% FDR, two unique peptides) (XLSX).

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

aSAH, aneurysmal subarachnoid hemorrhage; AUC, area under the ROC curve; CI, confidence interval; CID, collision-induced dissociation; CRP, C-reactive protein; CSF, cerebrospinal fluid; CT, computed tomography; ELISA, enzyme-linked immunosorbent assay; EVD, external ventricular drain; FDR, false discovery rate; GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Scale; GTP, guanosin-5-triphosphate; HCD, higher-energy collisional dissociation; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; ICU, intensive care unit; IPG, immobilized pH gradient; MSD, mesoscale discovery; OGE, off-gel electrophoresis; OT, OrbiTrap; ROC, receiver operating characteristic; SE, sensitivity; SMARTER, simultaneous marker discovery and verification for the rapid translation of exogenous reference material; SP, specificity; TEAB, tetraethylammonium bromide; TCEP, tris(2-carboxyethil) phosphine hydrochloride; TMT, tandem mass tag; WB, Western blot; WBC, white blood cells; WFNS, World Federation of Neurosurgical Societies

■ REFERENCES

(1) Suarez, J. I.; Tarr, R. W.; Selman, W. R. Aneurysmal Subarachnoid Hemorrhage. *N. Engl. J. Med.* **2006**, *354* (4), 387−396.

(2) Linn, F. H. H.; Rinkel, G. J. E.; Algra, A.; van Gijn, J. Incidence of Subarachnoid Hemorrhage: Role of Region, Year, and Rate of Computed Tomography: A Meta-Analysis. *Stroke* **1996**, *27* (4), 625−629.

(3) de Rooij, N. K.; Linn, F. H.; van der Plas, J. A.; Algra, A.; Rinkel, G. J. Incidence of subarachnoid haemorrhage: a systematic review with emphasis on region, age, gender and time trends. *J. Neurol., Neurosurg. Psychiatry* **2007**, *78* (12), 1365−72.

(4) Schievink, W. I. Intracranial Aneurysms. *N. Engl. J. Med.* **1997**, *336* (1), 28−40.

(5) Dirnagl, U.; Klehmet, J.; Braun, J. S.; Harms, H.; Meisel, C.; Ziemssen, T.; Prass, K.; Meisel, A. Stroke-Induced Immunodepression: Experimental Evidence and Clinical Relevance. *Stroke* **2007**, *38* (2), 770−773.

(6) Offner, H.; Vandenbark, A. A.; Hurn, P. D. Effect of experimental stroke on peripheral immunity: CNS ischemia induces profound immunosuppression. *Neuroscience* **2009**, *158* (3), 1098−111.

(7) Haeusler, K. G.; Schmidt, W. U. H.; Föhring, F.; Meisel, C.; Helms, T.; Jungehulsing, G. J.; Nolte, C. H.; Schmolke, K.; Wegner, B.; Meisel, A.; Dirnagl, U.; Villringer, A.; Volk, H. D. Cellular Immunodepression Preceding Infectious Complications after Acute Ischemic Stroke in Humans. *Cerebrovasc. Dis.* **2008**, *25* (1−2), 50−58.

(8) Sarrafzadeh, A.; Schlenk, F.; Meisel, A.; Dreier, J.; Vajkoczy, P.; Meisel, C. Immunodepression after aneurysmal subarachnoid hemorrhage. *Stroke* **2011**, *42* (1), 53−8.

(9) Frontera, J. A.; Fernandez, A.; Schmidt, J. M.; Claassen, J.; Wartenberg, K. E.; Badjatia, N.; Parra, A.; Connolly, E. S.; Mayer, S. A. Impact of nosocomial infectious complications after subarachnoid hemorrhage. *Neurosurgery* **2008**, *62* (1), 80−7.

(10) Ulm, L.; Ohlraun, S.; Harms, H.; Hoffmann, S.; Klehmet, J.; Ebmeyer, S.; Hartmann, O.; Meisel, C.; Anker, S. D.; Meisel, A. STRoke Adverse outcome is associated WIth NoSocomial Infections (STRAWINSKI): procalcitonin ultrasensitive-guided antibacterial therapy in severe ischaemic stroke patients - rationale and protocol for a randomized controlled trial. *Int. J. Stroke* **2013**, *8* (7), 598−603.

(11) Wartenberg, K. E.; Stoll, A.; Funk, A.; Meyer, A.; Schmidt, J. M.; Berrouschot, J. Infection after acute ischemic stroke: risk factors, biomarkers, and outcome. *Stroke Res. Treat.* **2011**, *2011*, 830614.

(12) Kammersgaard, L. P.; Jørgensen, H. S.; Reith, J.; Nakayama, H.; Houth, J. G.; Weber, U. J.; Pedersen, P. M.; Olsen, T. S. Early infection and prognosis after acute stroke: The Copenhagen Stroke Study. *Journal of Stroke and Cerebrovascular Diseases* **2001**, *10* (5), 217−221.

(13) Azurmendi, L.; Degos, V.; Tiberti, Natalia; Kapandji, Natacha; Sanchez, Paola; Sarrafzadeh, Asita; Puybasset, Louis; Turck, Natacha; Sanchez, Jean-Charles Neopterin plasma levels correlate with infection and long-term outcome in aneurysmal subarachnoid haemorrhage. *Journal of Neurosurgery - [Epub ahead of print]* **2015**, 150721213858005.

(14) Oconnor, E.; V, B.; Mashongonyika, C.; Lipman, J.; Hall, J.; Thomas, P. Serum Procalcitonin and C-reactive proteins as markers of sepsis and outcome in patients with neurotrauma and subarachnoid haemorrhage. *Anesth. Intensive Care* **2004**, *32* (4), 465−470.

(15) Westendorp, W. F.; Nederkoorn, P. J.; Vermeij, J. D.; Dijkgraaf, M. G.; van de Beek, D. Post-stroke infection: a systematic review and meta-analysis. *BMC Neurol.* **2011**, *11*, 110.

(16) Teasdale, G. M.; Drake, C. G.; Hunt, W.; Kassell, N.; Sano, K.; Pertuiset, B.; De Villiers, J. C. A universal subarachnoid hemorrhage scale: report of a committee of the World Federation of Neurosurgical Societies. *J. Neurol., Neurosurg. Psychiatry* **1988**, *51* (11), 1457.

(17) Teasdale, G.; Murray, G.; Parker, L.; Jennett, B. Adding up the Glasgow Coma Score. *Acta Neurochir Suppl* **1979**, *28* (1), 13−6.

(18) Claassen, J.; Bernardini, G. L.; Kreiter, K.; Bates, J.; Du, Y. E.; Copeland, D.; Connolly, E. S.; Mayer, S. A. Effect of Cisternal and

DOI: 10.1021/acs.jproteome.5b00391
J. Proteome Res. 2015, 14, 3948−3956

Ventricular Blood on Risk of Delayed Cerebral Ischemia After Subarachnoid Hemorrhage:: The Fisher Scale Revisited. *Stroke* **2001**, *32* (9), 2012−2020.
(19) Jennett, B.; Bond, M. Assessment of outcome after severe brain damage. *Lancet* **1975**, *1* (7905), 480−484.
(20) Calandra, T.; Cohen, J. The international sepsis forum consensus conference on definitions of infection in the intensive care unit. *Crit. Care Med.* **2005**, *33* (7), 1538−48.
(21) Dayon, L.; Turck, N.; Kienle, S.; Schulz-Knappe, P.; Hochstrasser, D. F.; Scherl, A.; Sanchez, J. C. Isobaric tagging-based selection and quantitation of cerebrospinal fluid tryptic peptides with reporter calibration curves. *Anal. Chem.* **2010**, *82* (3), 848−58.
(22) Dayon, L.; Turck, N.; Scherl, A.; Hochstrasser, D. F.; Burkhard, P. R.; Sanchez, J.-C. From Relative to Absolute Quantification of Tryptic Peptides with Tandem Mass Tags: Application to Cerebrospinal Fluid. *Chimia* **2010**, *64* (3), 132−135.
(23) Dayon, L.; Pasquarello, C.; Hoogland, C.; Sanchez, J.-C.; Scherl, A. Combining low- and high-energy tandem mass spectra for optimized peptide quantification with isobaric tags. *J. Proteomics* **2010**, *73* (4), 769−777.
(24) Gluck, F.; Hoogland, C.; Antinori, P.; Robin, X.; Nikitin, F.; Zufferey, A.; Pasquarello, C.; Fétaud, V.; Dayon, L.; Müller, M.; Lisacek, F.; Geiser, L.; Hochstrasser, D.; Sanchez, J.-C.; Scherl, A. EasyProt — An easy-to-use graphical platform for proteomics data analysis. *J. Proteomics* **2013**, *79* (0), 146−160.
(25) Elias, J. E.; Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* **2007**, *4* (3), 207−14.
(26) Breitwieser, F. P.; Muller, A.; Dayon, L.; Kocher, T.; Hainard, A.; Pichler, P.; Schmidt-Erfurth, U.; Superti-Furga, G.; Sanchez, J. C.; Mechtler, K.; Bennett, K. L.; Colinge, J. General statistical modeling of data from protein relative expression isobaric tags. *J. Proteome Res.* **2011**, *10* (6), 2758−66.
(27) Tiberti, N.; Hainard, A.; Lejon, V.; Robin, X.; Ngoyi, D. M.; Turck, N.; Matovu, E.; Enyaru, J.; Ndung'u, J. M.; Scherl, A.; Dayon, L.; Sanchez, J.-C. Discovery and Verification of Osteopontin and Beta-2-microglobulin as Promising Markers for Staging Human African Trypanosomiasis. *Mol. Cell. Proteomics* **2010**, *9* (12), 2783−2795.
(28) Tan, H. T.; Tan, S.; Lin, Q.; Lim, T. K.; Hew, C. L.; Chung, M. C. Quantitative and temporal proteome analysis of butyrate-treated colorectal cancer cells. *Mol. Cell. Proteomics* **2008**, *7* (6), 1174−85.
(29) Salvisberg, C.; Tajouri, N.; Hainard, A.; Burkhard, P. R.; Lalive, P. H.; Turck, N. Exploring the human tear fluid: discovery of new biomarkers in multiple sclerosis. *Proteomics: Clin. Appl.* **2014**, *8* (3−4), 185−94.
(30) van Bussel, B. C. T.; Henry, R. M. A.; Schalkwijk, C. G.; Ferreira, I.; Feskens, E. J. M.; Streppel, M. T.; Smulders, Y. M.; Twisk, J. W. R.; Stehouwer, C. D. A. Fish Consumption in Healthy Adults Is Associated with Decreased Circulating Biomarkers of Endothelial Dysfunction and Inflammation during a 6-Year Follow-Up. *J. Nutr.* **2011**, *141* (9), 1719−1725.
(31) Robin, X.; Turck, N.; Hainard, A.; Tiberti, N.; Lisacek, F.; Sanchez, J. C.; Muller, M. pROC: an open-source package for R and S + to analyze and compare ROC curves. *BMC Bioinf.* **2011**, *12* (77), 1471−2105.
(32) Langer, M.; Pifferi, S.; Peta, M. Diagnosis of bacterial infection in the ICU: General principles. *Intensive Care Med.* **1994**, *20* (4), S12−S16.
(33) Wartenberg, K. E.; Stoll, A.; Funk, A.; Meyer, A.; Schmidt, J. M.; Berrouschot, J. Infection after Acute Ischemic Stroke: Risk Factors, Biomarkers, and Outcome. *Stroke Res. Treat.* **2011**, *2011*, 830614.
(34) Fluri, F.; Morgenthaler, N. G.; Mueller, B.; Christ-Crain, M.; Katan, M. Copeptin, Procalcitonin and Routine Inflammatory Markers−Predictors of Infection after Stroke. *PLoS One* **2012**, *7* (10), e48309.
(35) Ruokonen, E.; Ilkka, L.; Niskanen, M.; Takala, J. Procalcitonin and neopterin as indicators of infection in critically ill patients. *Acta Anaesthesiol. Scand.* **2002**, *46* (4), 398−404.
(36) Hug, A.; Murle, B.; Dalpke, A.; Zorn, M.; Liesz, A.; Veltkamp, R. Usefulness of serum procalcitonin levels for the early diagnosis of stroke-associated respiratory tract infections. *Neurocrit. Care* **2011**, *14* (3), 416−22.
(37) Ihi, T.; Nakazato, M.; Mukae, H.; Matsukura, S. Elevated concentrations of human neutrophil peptides in plasma, blood, and body fluids from patients with infections. *Clin. Infect. Dis.* **1997**, *25* (5), 1134−40.
(38) Duthie, S.; Eckersall, P. D.; Addie, D. D.; Lawrence, C. E.; Jarrett, O. Value of alpha 1-acid glycoprotein in the diagnosis of feline infectious peritonitis. *Vet. Rec.* **1997**, *141* (12), 299−303.
(39) Kasvosve, I.; Speeckaert, M. M.; Speeckaert, R.; Masukume, G.; Delanghe, J. R. Haptoglobin polymorphism and infection. *Adv. Clin Chem.* **2010**, *50*, 23−46.
(40) Skinner, J. G.; Roberts, L. Haptoglobin as an indicator of infection in sheep. *Vet. Rec.* **1994**, *134* (2), 33−6.
(41) Malle, E.; De Beer, F. C. Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice. *Eur. J. Clin. Invest.* **1996**, *26* (6), 427−435.
(42) Epstein, F. H.; Gabay, C.; Kushner, I. Acute-Phase Proteins and Other Systemic Responses to Inflammation. *N. Engl. J. Med.* **1999**, *340* (6), 448−454.
(43) Nakayama, T.; Sonoda, S.; Urano, T.; Yamada, T.; Okada, M. Monitoring both serum amyloid protein A and C-reactive protein as inflammatory markers in infectious diseases. *Clin. Chem.* **1993**, *39* (2), 293−297.
(44) Cicarelli, D. D.; Vieira, J. E.; Bensenor, F. E. M.; Martins, b. E. Comparison of C-Reactive Protein and Serum Amyloid A Protein in Septic Shock Patients. *Mediators Inflammation* **2008**, *2008*, 1−5.
(45) Hultén, C.; Demmers, S. Serum amyloid A (SAA) as an aid in the management of infectious disease in the foal: comparison with total leucocyte count, neutrophil count and fibrinogen. *Equine Vet J.* **2002**, *347* (7), 693−698.
(46) Yuan, H.; Huang, J.; Lv, B.; Yan, W.; Hu, G.; Wang, J.; Shen, B. Diagnosis Value of the Serum Amyloid A Test in Neonatal Sepsis: A Meta-Analysis. *BioMed Res. Int.* **2013**, *2013*, 1−9.
(47) Arnon, S.; Litmanovitz, I.; Regev, R.; Lis, M.; Shainkin-Kestenbaum, R.; Dolfin, T. Serum amyloid A protein in the early detection of late-onset bacterial sepsis in preterm infants. *J. Perinat. Med.* **2002**, *30* (4), 329−32.
(48) Ilzecka, J.; Stelmasiak, Z. Prognostic Importance of Monitoring Serum Amyloid a Protein (SAA) In Patients with Cerebral Infarction. *Acta Clin. Croatica* **2000**, *39*, 139−146.
(49) Brämer, D.; Hoyer, H.; Günther, A.; Nowack, S.; Brunkhorst, F. M.; Witte, O. W.; Hoyer, D. Study protocol: prediction of stroke associated infections by markers of autonomic control. *BMC Neurol.* **2014**, *14* (9), 9.
(50) Emsley, H. C.; Smith, C. J.; Tyrrell, P. J.; Hopkins, S. J. Inflammation in acute ischemic stroke and its relevance to stroke critical care. *Neurocrit. Care* **2008**, *9* (1), 125−38.
(51) Widmer, A. F. Infection control and prevention strategies in the ICU. *Intensive Care Med.* **1994**, *20* (4), S7−S11.
(52) Harms, H.; Prass, K.; Meisel, C.; Klehmet, J.; Rogge, W.; Drenckhahn, C.; Göhler, J.; Bereswill, S.; Göbel, U.; Wernecke, K. D.; Wolf, T.; Arnold, G.; Halle, E.; Volk, H.-D.; Dirnagl, U.; Meisel, A. Preventive Antibacterial Therapy in Acute Ischemic Stroke: A Randomized Controlled Trial. *PLoS One* **2008**, *3* (5), e2158.
(53) Schwarz, S.; Al-Shajlawi, F.; Sick, C.; Meairs, S.; Hennerici, M. G. Effects of Prophylactic Antibiotic Therapy With Mezlocillin Plus Sulbactam on the Incidence and Height of Fever After Severe Acute Ischemic Stroke: The Mannheim Infection in Stroke Study (MISS). *Stroke* **2008**, *39* (4), 1220−1227.
(54) Lampl, Y.; Boaz, M.; Gilad, R.; Lorberboym, M.; Dabby, R.; Rapoport, A.; Anca-Hershkowitz, M.; Sadeh, M. Minocycline treatment in acute stroke: an open-label, evaluator-blinded study. *Neurology* **2007**, *69* (14), 1404−1410.
(55) Chamorro, A.; Horcajada, J. P.; Obach, V.; Vargas, M.; Revilla, M.; Torres, F.; Cervera, A.; Planas, A. M.; Mensa, J. The Early

DOI: 10.1021/acs.jproteome.5b00391
J. Proteome Res. 2015, 14, 3948−3956

Systemic Prophylaxis of Infection After Stroke Study: A Randomized Clinical Trial. *Stroke* **2005**, *36* (7), 1495−1500.
(56) Jin, R.; Yang, G.; Li, G. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *J. Leukocyte Biol.* **2010**, *87* (5), 779−789.

DOI: 10.1021/acs.jproteome.5b00391
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Chapter V

Infection prediction for aneurysmal subarachnoid hemorrhage patients at hospital admission: combined panel of serum amyloid A and clinical parameters

Infection prediction for aneurysmal subarachnoid hemorrhage patients at hospital admission: combined panel of serum amyloid A and clinical parameters

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In the previous article we have shown that SAA was a promising marker able to predict at hospital admission the aSAH patients that will develop an infection during their hospital stay. However, in the present chapter we have postulated that the accuracy of this acute phase molecule could be importantly improved by combining it with other individual parameters.

To find the best combination, we used 104 patients coming from two different cohorts of patients and the Panelomix tool, a method based in the iterative combination of biomarkers and thresholds. Among the different tested clinical scales (WFNS, GCS, age) and biomarkers (SAA, CRP, NP, WBC), the combination of SAA, WBC, WFNS, and age appeared to importantly improve the accuracy when comparing with single parameters. A specificity (SP) value of 100% was obtained for 64% sensitivity (SE), meaning that at hospital admission 6 out of 10 patients could be already classified as high-risk patients for infection.

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Infection prediction for aneurysmal subarachnoid hemorrhage patients at hospital admission: combined panel of serum amyloid A and clinical parameters

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Abstract

Purpose: Nosocomial infections are some of the main causes of worsening outcomes and death in aneurysmal subarachnoid hemorrhage patients. We hypothesize that combination of clinical parameters and blood biomarkers could increase the capacity of individual markers to dichotomize the patients at risk of infections.

Methods: The present study included 104 patients (69 infected/35 non-infected) from two independent European cohorts. Accuracy of biomarkers (serum amyloid A, C-reactive protein, neopterin and WBC) and clinical parameters (WFNS, GCS and age) were evaluated at hospital admission using receiver operating characteristic curves. The most accurate panel combination was obtained using Panelomix.

Results: At hospital admission, the most sensitive parameters for the stratification of patients at risk of developing an infection were SAA and the WFNS. To reach a SP of 100% (95% CI, 100–100), SE values of 26.9% (95% CI, 15.9–38.1) and 31.9% (95% CI, 21.7–43.5) were obtained respectively. Moreover, the combination of SAA, WBC, WFNS, and age significantly improved the SE to 64.3% (95% CI, 50–78.6).

Conclusions: At hospital admission the panel SAA, WBC, WFNS, and age appear as a promising tool for predicting in-hospital infections, which could lead to a better management of patients and in their associated outcomes.

Abbreviations: aSAH: aneurysmal subarachnoid hemorrhage; AUC: area under the ROC curve; CI: confidence interval; CRP: C-reactive protein; ELISA: enzyme-linked immunosorbent assay; GCS: Glasgow Coma Scale; GOS: Glasgow Outcome Scale; ICU: intensive care unit; MSD: mesoscale discovery; NP: neopterin; ROC: receiver operating characteristic; SAA: serum amyloid A; SE: sensitivity; SP: specificity; UTI: urinary tract infection; WBC: white blood cells; WFNS: World Federation of Neurosurgical Societies.

Introduction

Nosocomial infections are common complications in patients suffering from aneurysmal subarachnoid hemorrhage (aSAH). Pneumonia and urinary tract infections (UTI) are the most prevalent, developing in at least one third of aSAH patients [1,2]. Such infections can significantly impair patients' outcomes, prolonging hospital stays, and increasing associated rates of morbidity and mortality [3-5]. Identifying patients at a higher risk of developing an infection is crucial to promptly improve treatment for a potentially long-lasting disability [6,7].

Blood biomarkers can be effective risk assessment indicators; they can provide information about the disease's pathophysiology and, furthermore, they are objectively measurable throughout hospitalization [8,9]. In stroke patients, C-reactive protein (CRP) or white blood cells (WBC) are the two most commonly used infection markers [10,11]. However, for aSAH patients, no blood biomarkers are available in clinical practice. We recently showed that neopterin (NP) and serum amyloid A (SAA) could be promising predictive markers of infection as their plasma concentrations were significantly higher in patients developing infection during hospitalization compared to those who did not [12-14].

The utility of combining multiple parameters to improve the performance of individual markers has been described previously [15-19]. Therefore, in the present study, we postulated that combining previously described blood biomarkers with aSAH clinical parameters might be an efficient strategy for the stratification of patients in need of prophylactic antibiotherapy. Firstly, we evaluated how effectively different blood biomarkers (SAA, CRP, NP, and WBC) and clinical parameters (GCS, WNFS, Fisher, and age) at hospital admission were in identifying patients who subsequently developed an infection during hospitalization. Secondly, we tested which combination of parameters formed the best test panel for predicting the risk of infection.

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Materials and methods

Patient description

The present study included 104 patients from two different cohorts of patients: 25 patients admitted consecutively to the Charité Universitätsmedizin Hospital in Berlin (Germany) between January 2010 and August 2012 and 79 patients admitted consecutively to the Pitié-Salpêtrière Hospital in Paris (France) between July 2004 and April 2008.

The present study was approved by both hospitals' ethics committees: the Local Research Ethics Committee at Charité Virchow Medical Center and the Patient Protection Committee at the Pitié-Sapêtrière Hospital respectively. As per the Declaration of Helsinki, all the patients or the legal representatives signed an informed consent form.

The inclusion criteria for the present study were: 1) aged 18 years old or above, 2) aSAH confirmed by computed tomography and angiography, and 3) hospital admission within 48 hours of the onset of the hemorrhage. Patients presenting with more than one hemorrhagic event, not enough sample volume, and/or missing clinical information were excluded (Supplementary figure 1).

The severity of aSAH patients was assessed at hospital admission using the World Federation of Neurosurgical Societies scale (WFNS 1–5) [20]. Patients were dichotomized between good (WFNS 1–2) or poor neurological state (WFNS 3–5) depending on their initial clinical state. According to the Glasgow Coma Scale (GCS) patients were separated into three groups: patients with severe brain injury (GCS<9), moderate brain injury (GCS 9–12), and minor brain injury (GCS ≥ 13) [20,21]. The severity of the hemorrhage was established according to the Fisher score [22]: grade 1, no subarachnoid blood; grade 2, broad diffusion of subarachnoid blood; grade 3, with clots or thick layers of blood; grade 4, intraventricular hemorrhage or intracerebral hematoma, no clot; grade 5, intraventricular hemorrhage or intracerebral hematoma with clot.

The best type of intervention (clipping or coiling) was determined based on the location and size of each aneurysm.

The Glasgow Outcome Scale (GOS) was used to evaluate patients outcome one year after hospital admission. Depending on the level of functional dependence, patients were dichotomized into poor (GOS score of 1, 2, or 3) or good outcome groups (GOS score of 4 or 5) [23].

Infection status was established according to the International Sepsis Forum Consensus Conference on Definitions of Infection in the ICU [24]. Patients were classified as having or not this kind of infections: pneumonia, UTI or bloodstream infection. Bacteriological samples were taken at this time point, and the treatment was adjusted once results were obtained.

The demographic characteristics of the patients are shown in Table 1.

Marker measurement

Plasma samples were collected from patients on the day of hospital admission.

Concentrations of SAA, CRP, NP, and WBC were measured in these plasma samples collected on the day of hospital admission.

The Meso Scale Discovery (MSD) Vascular Injury Panel-I ECL assay was used to determine the levels of SAA and CRP in plasma, as per manufacturer's instructions (MSD, Gaithersburg, MD). Plasma samples were diluted 1:2000, and analyte concentrations were determined using an electrochemiluminescence detection system using multi-array technology (SECTOR Imager 2400, Meso Scale Discovery) [25].

NP levels in plasma were measured using a commercial ELISA kit, as per manufacturer's instructions (Brahms Gmbh, Hennigsdorf, Germany). Samples were diluted 1:4.

WBC count was determined using four-color flow cytometry on a FACSCalibur using CellQuest Software (BD Biosciences) [26].

All analytes were measured in duplicates (CV < 15%). Marker concentrations in the two different cohorts were normalized according to the median concentrations in each population set [27].

Table 1. Demographic characteristics of the population studied, comparing the presence or absence of infection during hospitalization.

104 Patient set			
	Infection (N=69)	**No infection (N=35)**	***P*[1]**
Gender			0.339
Male *n (%)*	22 (21.2%)	9 (8.7%)	
Female *n (%)*	47 (45.2%)	26 (25%)	
Age (years)			0.479
Average (± SD)	52.2 (± 12.4)	50.4 (± 12.1)	
WFNS score			**≤ 0.001**
1,2 *n (%)*	30 (28.8%)	28 (26.9%)	
3,4,5 *n (%)*	39 (37.5%)	7 (6.7%)	
Glasgow Coma Scale			**≤ 0.001**
< 9 *n (%)*	27 (26%)	2 (1.9%)	
9-12 *n (%)*	9 (8.7%)	2 (1.9%)	
≥ 13 *n (%)*	33 (31.7%)	31 (29.8%)	
Modified Fisher Score			**≤ 0.001**
0,1 *n (%)*	2 (1.9%)	11 (10.6%)	
2,3,4 *n (%)*	67 (64.4%)	24 (23.1%)	
Treatment			0.278
Surgery *n (%)*	8 (7.7%)	6 (5.8%)	
Embolisation *n (%)*	57 (54.8%)	29 (27.9%)	
No treatment *n (%)*	-	4 (3.8%)	
GOS 1 year			**0.007**
Good *n (%)*	31 (29.8%)	23 (22.1%)	
Poor *n (%)*	26 (25%)	4 (3.8%)	

Statistical data analysis

Statistical analyses were performed using SPSS software (version 21, SPSS Inc., Chicago, IL). As the levels of the different analytes were not normally distributed, Mann-Whitney U-test was used for statistical comparisons between two unpaired groups. Fisher's exact test and the Chi-squared test were used to assess whether the patients with and without infection were significantly different according to their gender, WFNS score, GCS, modified Fisher score, and outcome.

All statistical tests were bilateral, and a p-value <0.05 was considered statistically significant.

At hospital admission, receiver operating characteristic (ROC) curves were calculated for SAA, CRP, NP, and WBC as well as for the clinical parameters (GCS, WFNS, Fisher, and age). For each marker the SP value was restricted to 90-100% and the cut-off value was selected, in order to correctly classify 9 out of 10 patients that will not develop an infection. The pROC package for S+ (version 8.1., TIBCO Software Inc.) was used to calculate the values of areas under the partial area under the curve (AUC), specificity (SP), sensitivity (SE), and 95% confidence intervals (95% CI) [28].

Panel development

Panel selection was performed using the PanelomiX tool, as previously described [15]. Briefly, the optimized cut-off values were obtained by modified iterative permutation-response calculations (rule-induction-like, RIL) using all the individual parameters, the different analytes (SAA, CRP, NP, WBC) as well as the different clinical parameters (GCS, WFNS, Fisher, age). The cut-off values of each molecule or clinical parameter were changed iteratively by 2% increment quantiles. After each iteration the SE value was calculated using an SP value set between 90%–100%. The panel size was limited to a maximum of four parameters

Results

The demographic characteristics of the 104 patients included in the study are shown in Table 1. Most of the patients suffering from aSAH were women, however, the development of infection was unaffected by either gender or age. Neurological status at hospital admission and the severity of the hemorrhage were important factors affecting the development of the infection. More than 80% of patients with a poor neurological status and severe hemorrhage developed an infection during their hospital stay. Finally, there was a significant difference in patient outcome at one year between those patients' who had developed an infection and those who had not (Table 1).

Infection prediction according to blood biomarker concentrations and clinical parameters

In order to evaluate how effectively different blood biomarkers at hospital admission could predict which patients would later develop an infection, we measured the concentrations of SAA, CRP, NP, and WBC in the cohort.

As shown in Table 2, SAA, CRP, and WBC were significantly able to discriminate between patients who did and did not develop an infection. NP was the only biomarker unable to make this distinction at hospital admission.

AUC analyses were also performed on these four biomarkers (SAA, CRP, NP, and WBC) as well as on different clinical parameters (GCS, WFNS, Fisher, and age) in order to evaluate their accuracy in discriminating between patients with or without infection development.

SAA was the biomarker with the highest accuracy to differentiate between the groups of patients who did and did not develop an infection, reaching a SE value of 26.9% (95% CI, 15.9–38.1) for a SP value of 96.9 (95% CI, 90.6-100). The WFNS was the most effective clinical parameter, with SP values of 97.1 (95% CI, 91.4-100) and SE values of 31.9% (95% CI, 20.3-43.5) (Table 3). When the ROC analyses were performed on these molecules and clinical parameters to calculate the best combination of SP and SE, same results were found; SAA and WFNS were the two most accurate parameters (Supplementary table1).

Panel selection

To try to improve the accuracy for the prediction of infection, we assessed the possibility of combining all the parameters into panels. In order to select the most promising parameters for inclusion in the panel, we tested all the possible combinations of biomarkers. The iterative permutation-response approach used identified a four-parameter panel including SAA (cut-off: ≥ 427.4 µg/mL), WBC (cut-off: ≥ 13.7), WFNS score (cut-off: >3) and age (cut-off: ≥ 59). An accurate prediction was obtained when at least two of the four parameters were above the cut-off threshold value. The panel exhibited an SE of 64.3% (95% CI, 50–78.6) for an SP value of 100% (95% CI, 100–100) (Figure 1). This results in a significant increase in SE when compared to the best single biomarker (SAA) (p=0.003) and with the best clinical parameter (WFNS) (p=0.004), providing a significant improvement in the capacity to discriminate between patients who will and will not develop an infection.

Discussion

This prospective study involved 104 patients from two different cohorts. To the best of our knowledge, it is the first to have shown that, for aSAH patients, a predictive test panel comprising clinical parameters and biomarkers measured at hospital admission can significantly improve the prediction of infection compared to the use of single clinical parameters.

The early classification of patients at a higher risk of infection can be essential to improve the management of aSAH patients and their associated outcomes [29,30]. We therefore combined aSAH clinical parameters with known blood diagnostic biomarkers of infection (SAA, CRP, NP, and WBC) in order to find the most effective combination to predict the infection. The combination of SAA, WBC, WFNS, and age showed 100% SP (95% CI, 100–100) and 64.3% SE (95% CI, 50–78.6); interestingly all four parameters included in this panel had already been described individually as markers of infection [12,29,31,32].

The WFNS score, for instance, has been previously proposed as the most significant risk factor and predictor of infection in aSAH patients [1,31,33]. In line with these results, we showed that patients with high WFNS scores were more susceptible to developing an infection than patients with good neurological status ($p \leq 0.001$). This

Table 2. SAA, CRP, NP, and WBC median concentrations at hospital admission according to the presence or absence of infection.

104-Patient set			
	Infection (n = 69)	No infection (n = 35)	*P*
SAA (µg/mL)	181.03 (0-837.2)	46.42 (1.28-509.55)	**≤ 0.001**
CRP (µg/mL)	69.97 (0-362.95)	34.25 (0.77-217.2)	**0.001**
NP (nM)	10.1 (6.02-66.25))	9.5 (4.81-21.67)	0.157
WBC (million/mm^3)	13.1 (4.54-24.8)	11.62 (4.3-27.03)	**0.015**

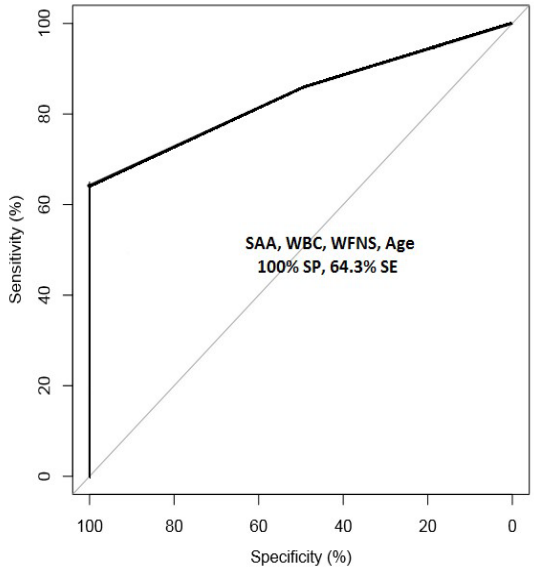

Figure 1. ROC curve of SAA, WBC, WFNS, and age test panel for the discrimination between infected and non-infected patients at hospital admission.

Table 3. Results of the ROC analysis assessing the capacity of different biomarkers and clinical parameters to differentiate between patients who did and did not develop an infection. The partial AUC, threshold, and SE correspond to SP set at 90%–100%.

Analytes				
SAA (μg/mL)	2.55 (1.44-5.29)	1021.42	96.9 (90.6 - 100)	26.56 (15.63 - 37.5)
CRP (μg/mL)	1.81 (0.79-4.17)	176.78	96.9 (90.6 - 100)	19.05 (9.52 - 28.57)
NP (nM)	1.06 (0.23-2.73)	14.59	97.14 (88.6 - 100)	10.14 (4.35 - 17.39)
WBC (million/mm^3)	0.76 (0-4.61)	Inf	100 (100 - 100)	0
Clinical Parameters				
GCS	2.95 (0.79-5.46)	6.5	97.14 (91.43 - 100)	31.88 (20.29 - 43.48)
WFNS	3.38 (1.62-5.6)	4.5	97.14 (91.43 - 100)	31.88 (21.74 - 43.48)
Fisher	2.46 (1.67-3.79)	4.5	100 (100 - 100)	14.49 (7.24 - 23.19)
Age	0.52 (0.04-2.17)	80	100 (100 - 100)	1.44 (0 - 4.35)

could be explained by the fact that ICU patients usually requiring more invasive procedures (mechanical ventilation, urinary and venous catheterization), are more likely to develop nosocomial infections. More importantly, these patients with high values of WFNS present a long suppression of the cellular immune response and an impaired pro-inflammatory response that increases the susceptibility of infection development [34].

It has also been suggested that patients' age correlates with the presence of pneumonia [5,17,29]. In the present cohort, all the different types of infection (pneumonia, UTI) were evaluated together, and it was not possible to determine the specific impact of pneumonia infections. Nevertheless, the mean age of patients' developing a nosocomial infection was higher than the age of those who did not, suggesting a positive trend toward the development of infections.

In addition to these two risk factors, the WBC count was also considered in panel generation. In aSAH patients the number of leukocytes have been correlated with the volume of the hemorrhage; being the patients with higher lesion and with higher levels of WBC, the patients with more complications and consequently with poorer outcome [32,35]. In the present study we found that, at hospital admission, patients who developed an infection had significantly higher levels of WBC than patients who did not. This reinforces the hypothesis that patients with poor neurological state at admission are susceptible to develop an infection.

The last parameter included in the panel was SAA—an acute phase inflammatory protein. This blood biomarker is of increasing interest due to its usefulness in monitoring and diagnosing a number of infections [36]. In neutropenic patients with acute leukemia, for example, SAA enabled a differentiation between infectious and non-infectious febrile episodes [37]. In children with a variety of viral infections, SAA concentrations increased during the acute phase of infection [38]. In a small number of aSAH patients it has also been already proposed as an infection marker [12]. In agreement with this, in the present study we have found that, at admission, SAA concentrations in patients who develop an infection were significantly higher than those who did not.

CRP and NP were the other two markers proposed as interesting infection predictors. Nevertheless, their accuracy to distinguish between the two groups of patients was not promising enough to be included in the panel.

CRP is close to SAA in terms of chemical properties; thus, their concentration levels usually follow the same trend across infectious diseases [39-41]. Accordingly, the present study found that, at hospital admission, CRP concentrations in patients developing an infection were significantly higher than in those who did not. Nevertheless, SAA was more accurate thus a more promising biomarker.

On the other hand, NP - a metabolite produced by monocytes/macrophages [42,43] - was initially described as an prognostic outcome biomarker for aSAH [44]. In a previous study, we showed also that NP correlates with the progression of infection from three days after hospitalization [14]. Nevertheless, present results showed that this discrimination capacity was not effective at the admission to the hospital, making from SAA an earlier and most accurate infection marker than NP.

This study showed that combining measurements of SAA, WBC, WFNS, and age significantly improved accuracy over the use of single markers or clinical parameters for the prediction of infection development in aSAH patients. Using the proposed test panel at least six out of ten patients who will develop an infection around five days following an aSAH event could be detected at hospital admission. The potential biochemical pathways occurring after a hemorrhagic event (leukocyte activation, coagulation systems, and complement systems, among others) are too variable and complex to be reflected by any one marker or clinical parameter. Therefore, the combination of biomarkers is more likely to correctly reflect this complex condition.

The panel here described has the potential to be translated, in the future, into an easy to use and interpret clinical tool. However, there are several issues which must be addressed before this important step.

Firstly, our results should be validated on other multicenter cohorts including a larger number of patients to obtain a robust confirmation of the data here presented.

The panel cut-off concentrations here established should be applied in a prospective study in order to confirm the accuracy of the test. Finally, in an effort to reduce the number of episodes of bacterial resistance, antibiotherapy should be reassessed daily; levels of biomarkers below which an infection is considered to be finished should be determined at the different times of hospitalization.

If all of these limitations can be properly addressed, then the prognostic panel presented here could be introduced into clinical practice as a promising tool to improve patient outcomes.

Conclusions

This two-center study showed that the combination of SAA, WBC, WFNS, and age at hospital admission significantly improved the accuracy of single parameters for the prediction of the infection development in aSAH patients. These promising results could ameliorate the management of patients presenting with aSAH in ICUs and their associated outcomes.

Conflict of interest

The authors declare no financial or other conflicts of interest related to this publication

Acknowledgments

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References

1. Frontera JA, Fernandez A, Schmidt JM, Claassen J, Wartenberg KE, et al. (2008) Impact of nosocomial infectious complications after subarachnoid hemorrhage. *Neurosurgery* 62: 80-87. [Crossref]

2. Hilker R, Poetter C, Findeisen N, Sobesky J, Jacobs A, et al. (2003) Nosocomial Pneumonia After Acute Stroke: Implications for Neurological Intensive Care Medicine. *Stroke* 34: 975-981. [Crossref]

3. Citerio G, Gaini SM, Tomei G, Stocchetti N, (2007) Management of 350 aneurysmal subarachnoid hemorrhages in 22 Italian neurosurgical centers. *Intensive Care Med* 33: 1580-1586. [Crossref]

4. Douds GL, Tadzong B, Agarwal AD, Krishnamurthy S, Lehman EB, et al. (2012) Influence of Fever and Hospital-Acquired Infection on the Incidence of Delayed Neurological Deficit and Poor Outcome after Aneurysmal Subarachnoid Hemorrhage. *Neurol Res Int* 2012: 6.

5. Laban KG, Rinkel GJE, Vergouwen MDI (2015) Nosocomial infections after aneurysmal subarachnoid hemorrhage: time course and causative pathogens. *Int J Stroke* 10: 763-766. [Crossref]

6. Ulm L, Ohlraun S, Harms H, Hoffmann S, Klehmet J, et al. (2013) STRoke Adverse outcome is associated WIth NoSocomial Infections (STRAWINSKI): procalcitonin ultrasensitive-guided antibacterial therapy in severe ischaemic stroke patients - rationale and protocol for a randomized controlled trial. *Int J Stroke* 8: 598-603. [Crossref]

7. Harms H, Prass K, Meisel C, Klehmet J, Rogge W, et al. (2008) Preventive Antibacterial Therapy in Acute Ischemic Stroke: A Randomized Controlled Trial. *PloS one* 3: e2158. [Crossref]

8. Mayeux R (2004) Biomarkers: Potential Uses and Limitations. *NeuroRx* 1: 182–188. [Crossref]

9. Hong CM, Tosun C, Kurland DB, Gerzanich V, Schreibman D, et al. (2014) Biomarkers as outcome predictors in subarachnoid hemorrhage--a systematic review. *Biomarkers* 19: 95-108. [Crossref]

10. Fluri F, Morgenthaler NG, Mueller B, Christ-Crain M, Katan M (2012) Copeptin, procalcitonin and routine inflammatory markers-predictors of infection after stroke. *PLoS One* 7: e48309. [Crossref]

11. Rallidis LS, Vikelis M, Panagiotakos DB, Rizos I, Zolindaki MG, et al. (2006) Inflammatory markers and in-hospital mortality in acute ischaemic stroke. *Atherosclerosis* 189: 193-197. [Crossref]

12. Azurmendi L, Degos V, Tiberti N, Kapandji N, Sanchez P, et al. (2015) Measuring Serum Amyloid A for Infection Prediction in Aneurysmal Subarachnoid Hemorrhage. J *Proteome Res* 14: 3948-3956. [Crossref]

13. Azurmendi L, Degos V, Tiberti N, Kapandji N, Sanchez P, et al. (2015) Neopterin plasma levels correlate with infection and long-term outcome in aneurysmal subarachnoid haemorrhage. *J Neurosurg* 124:1287-1299. [Crossref]

14. Azurmendi L, Degos V, Tiberti N, Kapandji N, Sanchez-Pena P, et al. (2015) Neopterin plasma concentrations in patients with aneurysmal subarachnoid hemorrhage: correlation with infection and long-term outcome. *J Neurosurg* 124: 1287-1299. [Crossref]

15. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, et al. (2013) PanelomiX: A threshold-based algorithm to create panels of biomarkers. *Translational Proteomics* 1: 57-64.

16. Turck N, Vutskits L, Sanchez-Pena P, Robin X, Hainard A, et al. (2010) A multiparameter panel method for outcome prediction following aneurysmal subarachnoid hemorrhage. *Intensive Care Med* 36: 107-115. [Crossref]

17. Hoffmann S, Malzahn U, Harms H, Koennecke HC, Berger K, et al. (2012) Development of a clinical score (A2DS2) to predict pneumonia in acute ischemic stroke. *Stroke* 43: 2617-2623. [Crossref]

18. Li Y, Song B, Fang H, Gao Y, Zhao L, et al. (2014) External validation of the A2DS2 score to predict stroke-associated pneumonia in a Chinese population: a prospective cohort study. *PLoS One* 9: e109665. [Crossref]

19. Albrich WC, Harbarth S (2015) Pros and cons of using biomarkers versus clinical decisions in start and stop decisions for antibiotics in the critical care setting. *Intensive Care Med* 41: 1739-1751. [Crossref]

20. Teasdale GM, Drake CG, Hunt W, Kassell N, Sano K, et al. (1988) A universal subarachnoid hemorrhage scale: report of a committee of the World Federation of Neurosurgical Societies. *J Neurol Neurosurg Psychiatry* 51: 1457. [Crossref]

21. Teasdale G, Murray G, Parker L, Jennett B (1979) Adding up the Glasgow Coma Score. *Acta Neurochir Suppl (Wien)* 28: 13-16. [Crossref]

22. Claassen J, Bernardini GL, Kreiter K, Bates J, Du YE, et al. (2001) Effect of Cisternal and Ventricular Blood on Risk of Delayed Cerebral Ischemia After Subarachnoid Hemorrhage:: The Fisher Scale Revisited. *Stroke* 32: 2012-2020. [Crossref]

23. Jennett B, Bond M (1975) Assessment of outcome after severe brain damage. *Lancet* 1: 480-484. [Crossref]

24. Calandra T, Cohen J (2005) The international sepsis forum consensus conference on definitions of infection in the intensive care unit. *Crit Care Med* 33: 1538-1548. [Crossref]

25. van Bussel BCT, Henry RMA, Schalkwijk CG, Ferreira I, Feskens EJM, et al. (2011) Fish Consumption in Healthy Adults Is Associated with Decreased Circulating Biomarkers of Endothelial Dysfunction and Inflammation during a 6-Year Follow-Up. *The Journal of Nutrition* 141: 1719-1725. [Crossref]

26. Klehmet J, Harms H, Richter M, Prass K, Volk HD, et al. (2009) Stroke-induced immunodepression and post-stroke infections: lessons from the preventive antibacterial therapy in stroke trial. *Neuroscience* 158: 1184-1193. [Crossref]

27. Yang YH, Dudoit S, Luu P, Lin DM, Peng V, et al. (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Research* 30: e15. [Crossref]

28. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, et al. (2011) pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 12: 77. [Crossref]

29. Harms H, Grittner U, Dröge H, Meisel A (2013) Predicting post-stroke pneumonia: the PANTHERIS score. *Acta Neurol Scand* 128: 178-184. [Crossref]

30. Grabska K, Gromadzka G, Czlonkowska A (2011) Infections and ischemic stroke outcome. *Neurol Res Int* 2011: 691348. [Crossref]

31. Savardekar A, Gyurmey T, Agarwal R, Podder S, Mohindra S, et al. (2013) Incidence, risk factors, and outcome of postoperative pneumonia after microsurgical clipping of ruptured intracranial aneurysms. *Surg Neurol Int* 4: 24. [Crossref]

32. Parkinson D, Stephensen S (1984) Leukocytosis and subarachnoid hemorrhage. *Surg Neurol* 21: 132-134. [Crossref]

33. Cinotti R, Dordonnat-Moynard A, Feuillet F, Roquilly A, Rondeau N, et al. (2014) Risk factors and pathogens involved in early ventilator-acquired pneumonia in patients with severe subarachnoid hemorrhage. *Eur J Clin Microbiol Infect Dis* 33: 823-830. [Crossref]

34. Sarrafzadeh A, Schlenk F, Meisel A, Dreier J, Vajkoczy P, et al. (2011) Immunodepression after aneurysmal subarachnoid hemorrhage. *Stroke* 42: 53-58. [Crossref]

35. Maiuri F, Gallicchio B, Donati P, Carandente M (1987) The blood leukocyte count and its prognostic significance in subarachnoid hemorrhage. *J Neurosurg Sci* 31: 45-48. [Crossref]

36. Urieli-Shoval S, Linke RP, Matzner Y (2000) Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states. *Curr Opin Hematol* 7: 64-69. [Crossref]

37. Casl MT, Rogina B, Glojnaric-Spasic I, Minigo H, Planinc-Peraica A, et al. (1994) The differential diagnostic capacity of serum amyloid A protein between infectious and non-infectious febrile episodes of neutropenic patients with acute leukemia. *Leuk Res* 18: 665-670

38. Miwata H1, Yamada T, Okada M, Kudo T, Kimura H, et al. (1993) Serum amyloid A protein in acute viral infections. *Arch Dis Child* 68: 210-214. [Crossref]

39. Nakayama T, Sonoda S, Urano T, Yamada T, Okada M (1993) Monitoring both serum amyloid protein A and C-reactive protein as inflammatory markers in infectious diseases. *Clinical Chemistry* 39: 293-297. [Crossref]

40. Lannergard A, Larsson A, Kragsbjerg P, Friman G (2003) Correlations between serum amyloid A protein and C-reactive protein in infectious diseases. *Scand J Clin Lab Invest* 63: 267-272. [Crossref]

41. Du Clos TW (2000) Function of C-reactive protein. *Ann Med* 32: 274-278. [Crossref]

42. Berdowska A, Zwirska-Korczala K (2001) Neopterin measurement in clinical diagnosis. *J Clin Pharm Ther* 26: 319-329. [Crossref]

43. Murr C, Widner B, Wirleitner B, Fuchs D (2002) Neopterin as a marker for immune system activation. *Curr Drug Metab* 3: 175-187. [Crossref]

44. Mathiesen T, Fuchs D, Wachter H, von Holst H (1990) Increased CSF neopterin levels in subarachnoid hemorrhage. *J Neurosurg* 73: 69-71. [Crossref]

Chapter VI

Proteomic discovery and verification of Serum Amyloid A, a predictor marker of patients at risk of post-stroke infection: a pilot study

Proteomic discovery and verification of Serum Amyloid A, a predictor marker of patients at risk of post-stroke infection: a pilot study

Azurmendi L*, Lapierre-Fetaud V.*, Schneider J., Montaner J., *Katan M., *Sanchez JC

In this chapter of the thesis, we have continued with our objective of finding a promising infection marker, however in this case instead of using aSAH patients, we have used a cohort of ischemic stroke patients. Similarly as reported in the chapter IV, we used proteomic quantitative analysis in infected and non-infected patients using a TMT-10plex to discover potential biomarkers. Among all the quantified proteins, the only significantly regulated one, SAA, was selected for further verification. The acute phase concentrations of this protein were evaluated using ELISA immunoassays in 44 additional patients.

These results confirmed that SAA could be a promising infection marker in patients with stroke, as its levels have shown to be elevated not only in patients suffering from aSAH but also from ischemic stroke.

This article has been published in *Clinical proteomics* in 2017. My contribution consisted in analyzing the data and writing the article.

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Proteomic discovery and verification of serum amyloid A as a predictor marker of patients at risk of post-stroke infection: a pilot study

L. Azurmendi[1†], V. Lapierre-Fetaud[1†], J. Schneider[2], J. Montaner[3], M. Katan[2†] and Jean-Charles Sanchez[1*†]

Abstract

Background: Post-stroke infections occur in 20–36% of stroke patients and are associated with high morbidity and mortality rates. Early identification of patients at risk of developing an infection could improve care via an earlier treatment leading to a better outcome. We used proteomic tools in order to discover biomarkers able to stratify patients at risk of post-stroke infection.

Methods: The post hoc analysis of a prospective cohort study including 40 ischemic stroke patients included 21 infected and 19 non-infected participants. A quantitative, isobaric labeling, proteomic strategy was applied to the plasma samples of 5 infected and 5 non-infected patients in order to highlight any significantly modulated proteins. A parallel reaction monitoring (PRM) assay was applied to 20 additional patients (10 infected and 10 non-infected) to verify discovery results. The most promising protein was pre-validated using an ELISA immunoassay on 40 patients and at different time points after stroke onset.

Results: Tandem mass analysis identified 266 proteins, of which only serum amyloid A (SAA1/2) was significantly ($p = 0.007$) regulated between the two groups of patients. This acute-phase protein appeared to be 2.2 times more abundant in infected patients than in non-infected ones. These results were verified and validated using PRM and ELISA immunoassays, which showed that infected patients had significantly higher concentrations of SAA1/2 than non-infected patients at hospital admission, but also at 1, 3, and 5 days after admission.

Conclusions: The present study demonstrated that SAA1/2 is a promising predictor, at hospital admission, of stroke patients at risk of developing an infection. Further large, multicenter validation studies are needed to confirm these results. If confirmed, SAA1/2 concentrations could be used to identify the patients most at risk of post-stroke infections and therefore implement treatments more rapidly, thus reducing mortality.

Background

Stroke is a serious medical condition produced by brain cell death. It occurs when there is a lack of blood flow to the brain (~80% of cases) or a hemorrhage affecting the brain or its surroundings (20%). Every year, around 15 million people will suffer a stroke, leading to 6 million deaths and 5 million disabled patients [1–3]. Around 40% of patients die within the first weeks following the stroke [4, 5]. Non-modifiable factors, such as the severity of the stroke or the age of the patient, are highly correlated with mortality [6, 7]. However, one-third of deaths result from potentially preventable stroke-associated complications. Nosocomial infection, particularly bacterial pneumonia, is the most common complication after stroke, with an incidence of 5–22% [8–10]. Despite the intensive care given to these patients, infection rates remain elevated and are

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associated with bad functional outcome and mortality [11]. The high incidence of infection is likely to be a result of an impaired immune function. The patient's reduced ability combat bacteria is a consequence of the initial brain damage [12, 13]. Therefore, the early identification of patients who might be prone to developing an infection after stroke is a necessary step towards better hospital management, more rapid implementation of treatments, and improved long-term patient outcomes [13, 14].

In clinical practice, the diagnosis of post-stroke infection is a challenging one as there has been no satisfactory concordance between different studies. The most widely studied markers of post-stroke infection—procalcitonin (PCT), C-reactive protein (CRP), and white blood cells (WBC)—have only shown moderate predictive value, and their levels do not increase early enough to be of help before the infection is clinically apparent [15, 16]. Clinical signs such as older age, fever, severe stroke, or dysphagia, among others, have been linked to post-stroke associated pneumonia [17]. Nevertheless, they are not specific enough to act as individual markers. Using a combination of these markers with clinical scales such as the A2DS2, AIS-APS, and ISAN [17–19] have not been applied routinely in clinical practice. The gold standard for diagnosing an infection is the result from a bacterial culture, yet this may take 2 days. All of these reasons can lead to antibiotic treatment being started too late, with the unfortunate associated consequences. There is thus evidence of a need for a reliable early biomarker [20].

The present study aimed to use proteomic approaches to find a biomarker that could be tested for at hospital admission in order to identify patients at risk of developing a post-stroke infection. To do this, we investigated the plasma proteomes of infected and non-infected patients, using isobaric labeling methods. After selecting SAA1/2 as the most promising protein, parallel reaction monitoring (PRM) and the enzyme-linked immunosorbent assays (ELISA) confirmed its ability to predict which patients were at risk of infection after a stroke.

Methods

Study design and setting

We performed a post hoc analysis of a prospective cohort study which included 40 ischemic stroke patients (ClinicalTrials.gov.NCT00390962) who had been hospitalized consecutively at the University Hospital of Basel (Switzerland) between November 2006 and November 2007. The study protocol was conducted according to the principles expressed in the Declaration of Helsinki and with the approval of the local ethics committee. Before enrolment, informed consent was obtained from patients, their relatives, or their legal guardians.

Clinical protocol

Comprehensive information on the assessment of the study participants' demographic and vascular risk factors has been published previously [15]. Briefly, ischemic stroke was defined according to the World Health Organization criteria [21]. A detailed history was obtained for vascular risk factors, vital signs, and relevant comorbidities as assessed using the Charlson Comorbidity Index (CCI) medication taken prior to the stroke. Neurological deficits were estimated using the National Institutes of Health Stroke Scale (NIHSS). Patients underwent the following standardized diagnostic workup: brain computer tomography (CT) and/or magnetic resonance imaging, long-term electrocardiography, echocardiography, and neurosonographic imaging of the extracranial and intracranial arteries. Stroke etiology was determined according to the TOAST (Trial of Org 10172 in Acute Stroke Treatment) classification criteria, which distinguish large-artery arteriosclerosis, cardio embolism, small-artery occlusion, other etiologies, and undetermined etiologies [22].

Definition of stroke-associated Infections

Stroke-associated infection (SAI) was defined as any infection occurring within the first 5 days after hospital admission [13]. Infections were diagnosed according to the U.S. Centers for Disease Control and Prevention (CDC) criteria [15]. We distinguished between pneumonia, urinary tract infection (UTI), and other infections (OI). Pneumonia was diagnosed when at least one symptom from each of the two following symptom groups was present: (1) abnormal respiratory examination, pulmonary infiltrates in chest X-rays; (2) productive cough with purulent sputum, positive microbiological cultures from the lower respiratory tract or blood cultures.

Diagnosis of a UTI required two of the following criteria to be met: fever ($\geq$38.0 °C), urine sample positive for nitrite, leukocyturia ($\geq$40/μL), or significant bacteriuria ($\geq 10^4$/mL of an uropathogen). OI were diagnosed if white blood cell count was $\geq$11,000/mL and CRP was $\geq$10 mg/L or temperature was $\geq$38.0 °C and an infectious manifestation was present. The treating physician made the diagnosis of pneumonia during hospitalization. This was then validated post hoc using charts.

The time point of diagnosis was taken to be the beginning of clinical symptoms which led to the diagnostic workup and resulted in the diagnosis of infection. In order to exclude any acute infections that had preceded the stroke, patients with an admission temperature >38 °C, reporting an infection lasting up to 3 days before the onset of stroke, or who required mechanical intubation were not included in the study.

Blood sample collection

Blood samples were collected from venous blood puncture within the first 72 h following symptom onset and then 1, 3, and 5 days after admission. Blood was centrifuged for 30 min at 3000×*g*, collected in EDTA, tubes and stored at −80 °C.

Proteomic study

Quantitative proteomic analysis: TMT

Quantitative proteomic analyses were performed on five infected and five non-infected patients at hospital admission. The aim was to identify significantly regulated proteins between the two groups in order to find a promising infection marker.

Reduction, alkylation, digestion, and TMT labeling The quantitative proteomic experiment used 1 µL of each plasma sample. These amounts were dried and reconstituted in 16.6 µL of 6 M urea in tetraethylammonium bromide (TEAB) 0.1 M. The proteins were reduced by adding 1 µL of 50 mM tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) to each sample, and they then reacted for 1 h at 37 °C. After the sample had cooled to room temperature, the alkylation step required mixing the solution with 1 µL of iodoacetamide 400 mM and storage at room temperature for 30 min. Sixty seven µL of TEAB 0.1 M were added to reduce the concentration of urea to <2 M. The digestion was carried out overnight at 37 °C using 1 µg of trypsin for each 20 µg of protein. The protocol is detailed by Dayon et al. [23, 24].

Subsequently, each digested plasma sample was labeled with one of the 10 TMT reagents (Thermo Fisher Scientific, Waltham, USA). Infected patients' samples were labeled with TMTs 127n, 128n, 129n, 130n, and 131n. Non-infected patients' samples were labeled with TMTs 126, 127c, 128c, 129c, and 130c. To calculate experimental error, 1 µg of β-lactoglobulin was spiked in each sample. All the samples were pooled, desalted using a C18 Macro SpinColumn, and dried in a speed-vacuum.

Off-gel electrophoresis (OGE) OGE separation was carried out using an Agilent 3100 Off-Gel fractionator, as per the manufacturer's instructions. Previously dried samples were reconstituted using the OGE solution and then focused using an immobilized pH gradient (IPG) dry strip (13 cm, pH 3–10) [23]. After OGE, samples were desalted using a C18 Micro SpinColumn, dried in the speed-vacuum, and stored at −20 °C until analysis.

LC–MS/MS A Q-Exactive Plus mass spectrometer (ThermoFisher, San Jose, CA), coupled with nanoflow high-pressure liquid chromatography (HPLC), was used to analyze the OGE fractions, as previously described [25]. Briefly, peptides reconstituted using 5% CAN, 0.1% FA, were trapped in a 5 µm 200 Å Magic C18 AQ (Michrom) 0.1 × 20 mm pre-column and separated in a 5 µm 100 Å Magic C18 AQ (Michrom) 0.75 × 150 mm column with a gravity-pulled emitter. Both columns were made in-house. The analytical separation ran for 65 min using a gradient of H_2O/FA 99.9/0.1% (solvent A) and CH_3CN/FA 99.9/0.1% (solvent B). The gradient ran at a flow rate of 220 nL/min as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min. For the MS survey scans, OT resolution was set to 60,000 and the ion population was set to 5 × 105 with an m/z window from 400 to 2000. A maximum of three precursors were selected for both collision-induced dissociation (CID) in the LTQ and higher-energy collisional dissociation (HCD) with analysis in the OT. For MS/MS in the LTQ, the ion population was set to 7×10^3 (isolation width of 2 m/z), whereas for MS/MS detection in the OT, it was set to 2×10^5 (isolation width of 2.5 m/z), with a resolution of 7500, a first mass at m/z = 100, and a maximum injection time of 750 ms. The normalized collisional energies were set to 35% for CID and 60% for HCD.

Protein identification MS data were processed using EasyProtConv. Peak lists were obtained using the 12 OGE fractions and the combination of HCD-CID raw data peak lists were generated. Afterwards, these data were submitted to an EasyProt software platform (version 2.3, build 718) that uses Phenyx software (GeneBio, Geneva, Switzerland) for protein identification. The protein search was made using the Uniprot/Swiss-Prot database (2014–10, 669903) [26], applying the following search criteria: *Homo sapiens* taxonomy, oxidized methionine (as the variable modification), and cysteine carbamethylation, TMT^{10} lysine, and TMT^{10} amino-terminus (as the fixed modifications). Trypsin was selected as the proteolytic enzyme, allowing one missed cleavage. Parent-ion tolerance was set to 10 ppm and the accuracy of fragment ions to 0.6 Da. Only proteins with a less than 1% false discovery rate (FDR) and at least two different unique peptides were selected for further analysis [27]. A minimum peptide length of 6 amino acids was used.

Protein quantification used the Isobar R package [28]. The manufacturer's isotopic distribution data was used to correct the isotopic impurities of TMT^{10} reporter-ion intensities. The equal median intensity method was used to normalize the reporter intensities. Peptides which did not present reporter intensities were not quantified. The infection/no infection ratio was calculated for each peptide, combining the reporter-ion intensities between infected patient channels (127n, 128n, 129n, 130n, and 131n) and non-infected patient channels (126, 127c, 128c, 129c, and 130c). To test the ratio's accuracy and biological significance, technical and biological variability

were calculated for each protein ratio. A ratio *p* value and sample *p* value were calculated for each variable. Furthermore, only proteins with a cut-off threshold value higher than 1.5 or lower than 0.67 were considered [29–31].

SAA1/2 PRM analysis

Parallel reaction monitoring (PRM) analysis was performed on ten infected and ten non-infected plasma samples using a Q-Exactive Plus mass spectrometer (ThermoFisher), as previously described [32]. The aim was to verify the discovery results.

Each sample was loaded into a PepMap precolumn (2 cm × 75 µm i.d., C18, 3 µm, and 100 Å pore size). Subsequent separation was performed in a PepMap column (50 cm × 75 µm i.d., C18, 2 µm, 100 Å pore size). A mixture of mobile A and B phases was used for peptide elution. The phase A solvent was composed of 0.1% (v/v) formic acid (Biosolve) and HPLC-grade water (Romil); the phase B solvent was composed of 0.1% (v/v) formic acid in HPLC-grade acetonitrile (Romil). To perform the separation, a linear gradient of 5–35% solvent B at 250 nL/min for 60 min was set and it was followed by a washing step (35–90% of solvent B for 10 min).

Three masses were targeted (doubly and triply charged ions), corresponding to total SAA, but also specifically to SAA1 and SAA2. The selection of the different peptides was performed considering two different criteria: a previous SAA PRM study and the results of our quantitative proteomic analysis [32]. The three peptides selected in this way were tryptic peptides associated to each isoform.

This inclusion list triggered targeted scans at a resolving power of 70,000, with an isolation width of 1 Th around the m/z of interest, an AGC target of 1×10^6, a maximum injection time of 100 ms, and a normalized collision energy of 27% in a higher-energy c-trap dissociation (HCD) cell.

Data analysis Data were analyzed using the targeted MS/MS feature available in Skyline v3.5 software [33]. In order to confirm the identity of the peptides, a data dependent acquisition spectral library of annotated reference MS/MS spectra was created from the two pools of plasma samples composed of infected and non-infected patients. Peptides were quantified by extracting the peak areas of accurate fragment ions (<6 ppm), and they were then integrated across the peptides' elution profiles. For each peptide, transition peak areas were normalized by the average of the sum of the transition peak areas for all the peptides across the runs.

SAA1/2 ELISA measurement

The Vascular Injury Panel-I electrochemiluminescence (ECL) assay was used to determine the levels of SAA1/2 in 40 stroke patients, as per the manufacturer's instructions (Meso Scale Discovery, Gaithersburg, MD). Each plasma sample was diluted 1:1000 with using sample diluent provided by the kit. An ECL detection system using multi-array technology (SECTOR Imager 2400, Meso Scale Discovery) was used to determine analyte concentrations. Samples were measured in a single detection.

Statistical analyses

Statistical analyses were carried out using SPSS software (v21, SPSS Inc., Chicago, IL). Analytes were not normally distributed, so the Mann–Whitney U-test was used to compare the two unpaired groups. Fisher's exact test and the Chi squared test were used to assess whether patients with and without infection were significantly different according to their gender, medical history, clinical data, laboratory values, lesion size, or TOAST. All statistical tests were two-tailed, and a p value <0.05 was considered statistically significant.

Multivariate analyses were performed to assess the associations between variables. The presence/absence of infection was set as the dependent variable, and SAA, CRP, WBC, and NIHSS were set as confounders. The model was validated using the bootstrap method. Categorical data were dichotomized according to the criteria in the table of demographic characteristics. Longitudinal data were also dichotomized according to the best cut-off obtained from area under the receiver operating characteristic (ROC) curve (AUC) analysis.

Results

Baseline population characteristics

Of 40 consecutively enrolled ischemic stroke patients, 21 developed an infection within 5 days of stroke onset (day 4 was the median day of infection development after the cerebrovascular event). Mean patient age was 79 years old (IQR: 70–82 years) and 55% of patients were men. Patients with severe strokes, resulting in higher NIHSS values at hospital admission, were more prone to developing an infection than patients with minor strokes. Other factors, such as hypertension, diabetes mellitus, or smoking, did not significantly affect the development of an infection. Nevertheless, according to the modified Rankin Scale, patient outcome appeared to be significantly affected by the development of an infection, as most of the patients with a poor outcome had developed an infection during their hospital stay.

At hospital admission, levels of WBC and CRP were within the normal range in both groups, with no significant differences found between infected and non-infected patients. Patients' demographic characteristics are summarized in Table 1.

Table 1 Baseline data

	All patients ($n = 40$)	No infection ($n = 19$)	Infection ($n = 21$)	*p* value
Demographic data				
Age, median (IQR)	79.2 (70.4–82)	78.3 (74–80.5)	80.4 (69.5–83)	0.78
Female sex, *n* (%)	18 (45)	9 (47.4)	9 (42.9)	1
Medical history, *n* (%)				
Hypertension	31 (77.5)	12 (63.2)	19 (90.5)	0.06
Atrial fibrillation	9 (22.5)	3 (15.8)	6 (28.6)	0.17
Current smoking	11 (27.5)	5 (26.3)	6 (28.6)	0.64
Diabetes mellitus	7 (17.5)	4 (21.1)	3 (14.3)	0.69
Coronary heart disease	10 (25)	4 (21.1)	6 (28.6)	0.72
Previous stroke	11 (27.5)	5 (26.3)	6 (28.6)	1
Clinical data, median (IQR)				
NIHSS at admission	5.5 (2–12)	3 (2–7)	12 (4–14)	0.01
Laboratory values, median (IQR)				
WBC (g/l)	8.6 (6.8–10.1)	7.7 (6.2–9.3)	9.3 (7.4–11.2)	0.14
CRP (mg/l)	3.6 (3–9.1)	3.6 (3–6.8)	4.8 (3–17.4)	0.22
Lesion size on MR, DWI[b]				
Small (1–10 mm^3)	23 (27.5)	13 (68.4)	10 (47.6)	0.47
Medium (10–100 mm^3)	8 (20)	3 (15.8)	5 (23.8)	0.75
Large (>100 mm^3)	1 (2.5)	0 (0)	1 (4.8)	0.69
TOAST				
Large vessel stroke	8 (20)	3 (15.8)		0.69
Cardioembolic stroke	8 (20)	4 (21.1)		1
Microangipathic stroke	14 (35)	5 (26.3)		0.33
Other	0 (0)	0 (0)		
Unknown	10 (25)	7 (36.8)		0.15

Proteomic results

In order to find a biomarker able to distinguish, at hospital admission, which patients will and will not develop a post-stroke infection, the proteomes of five infected and five non-infected stroke patients were compared using quantitative proteomic analysis. Applying the criteria of a maximum of 1% FDR and at least two unique peptides, 266 proteins were quantified (Additional file 1: Table 1). Of all the proteins, serum amyloid A1 appeared to be the only significantly ($p = 0.007$) regulated protein between the two groups of patients, with a ratio of 2.2 after Bonferroni correction.

To verify the results obtained by the TMT^{10} plex during the discovery phase, a further PRM analysis was performed on a new batch of patients. Consequently, we targeted three transitions of the tryptic SFFSFLGEAFDGAR peptide in 10 infected and 10 non-infected patients. This peptide is common to all the different isoforms of acute-phase SAA. By measuring its concentration, therefore, we were sure to measure the total amount SAA present in blood and not only that of one of the different described isoforms. As shown in Fig. 1, the concentration of SFFSFLGEAFDGAR was significantly higher ($p < 0.001$) in infected patients than in non-infected ones, confirming that there was a clear overproduction of SAA in patients who went on to develop an infection.

Different SAA isoforms for infection development

Further PRM analyses were performed on the same 20 patients in order to evaluate whether either of the acute phase isoforms (SAA1 and SAA2) had a more significant effect on infection and inflammatory processes. The high sequence-similarity between the SAA1 and SAA2 isoforms prevented an evaluation of their effects using classic ELISAs. The present study measured three transitions in the FFGHGAEDSLADQAANEWGR peptide (unique to SAA1) and GPGGAWAAEVISNAR peptide (unique to SAA2) across 10 infected and 10 non-infected patients. As Additional file 2: Fig. 1 shows, both peptides were significantly ($p < 0.001$) more abundant in infected patients than in non-infected ones.

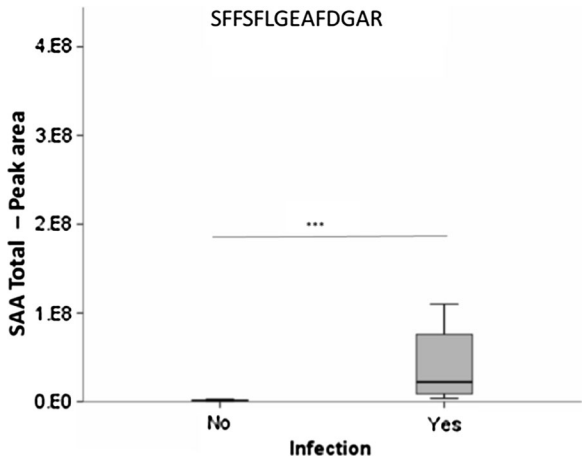

Fig. 1 Ratio of SFFSFLGEAFDGAR peptide abundance between patients who went on to develop an infection and those who did not. The SFFSFLGEAFDGAR peptide is common to all the different isoforms of SAA

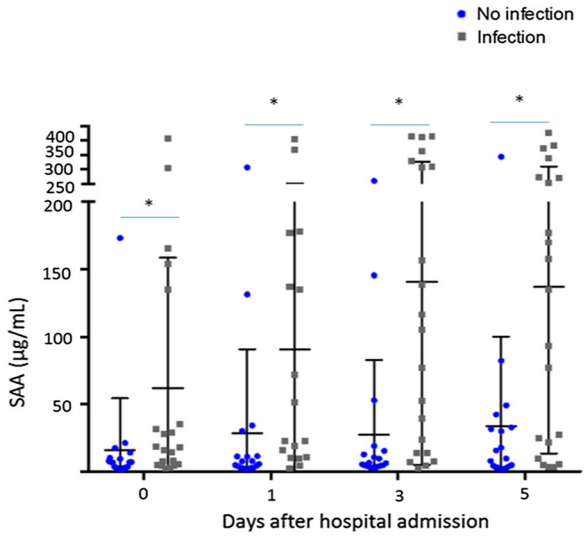

Fig. 2 Kinetics of SAA concentrations at hospital admission, 1, 3, and 5 days after stroke onset. Text under Fig. 2 in PDF: SAA concentrations are shown by *grey square* for infected patients and by *blue square* for non-infected patients. Comparisons between the two groups were made using the Mann–Whitney *U* test. *Asterisk* significance level reported after the Bonferroni correction ($p < 0.05$)

Kinetics of serum amyloid A1/2

Serum amyloid A1/2 plasma concentrations were subsequently measured in a new group of 21 infected and 19 non-infected patients in order to validate the previous proteomic results. Concentrations of this acute-phase reactant molecule were measured at hospital admission and at 1, 3, and 5 days after hospitalization, using an SAA1/2 ELISA assay. Initially, analyses were performed separately in those patients used for the discovery step and in those used for the verification/validation step. As Additional file 3: Fig. 2 shows, in both cases, SAA concentrations were significantly higher in patients who went on to develop a post-stroke infection than in those who did not. Subsequently, analyses were performed again when all the patients were evaluated together. As Fig. 2 shows, peptide concentrations were again significantly higher in infected patients than in non-infected patients, at all time points, particularly at 3 days ($p = 0.01$) and 5 days ($p = 0.01$) after stroke onset.

SAA measurements were evaluated to distinguish between the two groups of patients at D0, D1, D3, and D5. As Table 2 shows, the accuracy of SAA measurements in distinguishing which patients went on to develop an infection and which did not reached values of 73.2% (cut-off: 14.2 µg/mL) and 77.1% (cut-off: 8.8 µg/mL) at hospital admission and 1 day after, respectively. Three days after hospitalization, the AUC of SAA was slightly better, reaching a value of 80.7% (cut-off: 21.4 µg/mL), and 5 days after hospitalization, the AUC was 76.7% (cut-off: 87.7 µg/mL).

To evaluate the capacity of SAA1/2 measurement to rule-in patients at risk of infection, we set specificity (SP) at between 90 and 100%. At hospital admission, with a 94.7% SP, SAA measurement reached 42.9% sensitivity (SE) and a partial AUC of 2.5% (Table 2). Three days after hospitalization, SP reached 100%, SE was 33.3%, and the partial AUC was 3.6% (Table 2). All the AUC and pAUC curves obtained at the different time points are represented in Fig. 3. These AUC and pAUC values were obtained using different cut-off concentrations corresponding to the best combination of SP and SE.

However, due to the high variability of the SAA concentrations obtained, we decided to evaluate the possibility of using a ratio based on those concentrations to predict the development of an infection. As Table 2 shows, patients who went on to develop an infection during their hospital stay, presented with an average 2.4 times greater concentration of SAA on D3 than on D1. For patients who did not become infected, average SAA concentrations remained very similar (ratio of 0.97), with no significant increase, thus suggesting that this ratio could be used as an indicator of patients at risk.

Multivariate analyses

Finally, we performed multivariate analyses in order to confirm that SAA was a promising biomarker of post-stroke infection and to assess whether it was an independent predictive factor. The presence of infection was set as the dependent variable, and the significantly regulated parameters according to the patients' demographic characteristics (NIHSS and SAA) were set as confounders. WBC and CRP were also included in the confounder group because they are widely used in clinical practice.

Table 2 Capacity of plasma concentrations of SAA to distinguish between patients who went on to develop an infection and those who did not

Day	Number of patients		Mean SAA concentration (μg/mL) ± SD			ROC curve			
	No infection	Infection	No infection	Infection	p value	AUC (95% CI)	Cut-off	SP% (95% CI)	SE% (95% CI)
						pAUC (95% CI)		SP 90–100% (95% CI)	
0	19	21	16 ± 38.4	61.8 ± 96.7	0.01	73.2 (55.9–87)	14.2	84.2 (68.4–100)	61.9 (38.1–81)
						2.53 (0–6.7)	24.7	94.7 (84.2–100)	42.9 (23.8–62.02)
1	19	17	28.2 ± 62.6	57.7 ± 92.9	0.005	77.1 (60.1–92)	8.8	63.2 (42.1–84.2)	88.2 (70.6–100)
						2.3 (0.6–6.5)	133.4	94.7 (84.2–100)	35.3 (11.8–58.8)
3	19	21	27.6 ± 55.2	140.9 ± 136	0.001	80.7 (66.2–93.2)	21.4	84.2 (68.4–100)	71.4 (52.4–90.5)
						3.6 (1.7–7.6)	233.9	100 (100–100)	33.3 (14.3–52.4)
5	19	21	33.6 ± 66.4	137 ± 123.5	0.003	76.7 (61.2–89.7)	87.8	94.7 (84.2–100)	57.1 (38.1–76.2)
						3.5 (0.5–7.6)	87.8	94.7 (84.2–100)	57.1 (38.1–76.2)

As Table 3 shows, SAA was the only marker that displayed a relationship with the development of post-stroke infections, thus confirming and validating the possibility of measuring SAA concentrations as a biomarker of infection in stroke patients.

Discussion

The present study highlighted the capacity of proteomics to identify protein biomarkers that could assist in the detection of stroke patients at a high risk of developing post-stroke infection [34]. Using isobaric labeling methods, we first compared the plasma samples of five infected stroke patients and five non-infected stroke patients. We found that concentrations of serum amyloid A1 were overexpressed in patients who went on to develop an infection. This first approach was then verified using parallel reaction monitoring in 20 stroke patients (10 infected and 10 non-infected). Finally, the SAA1/2 concentrations of 40 ischemic stroke patients were confirmed using ELISA kits. The results demonstrated that

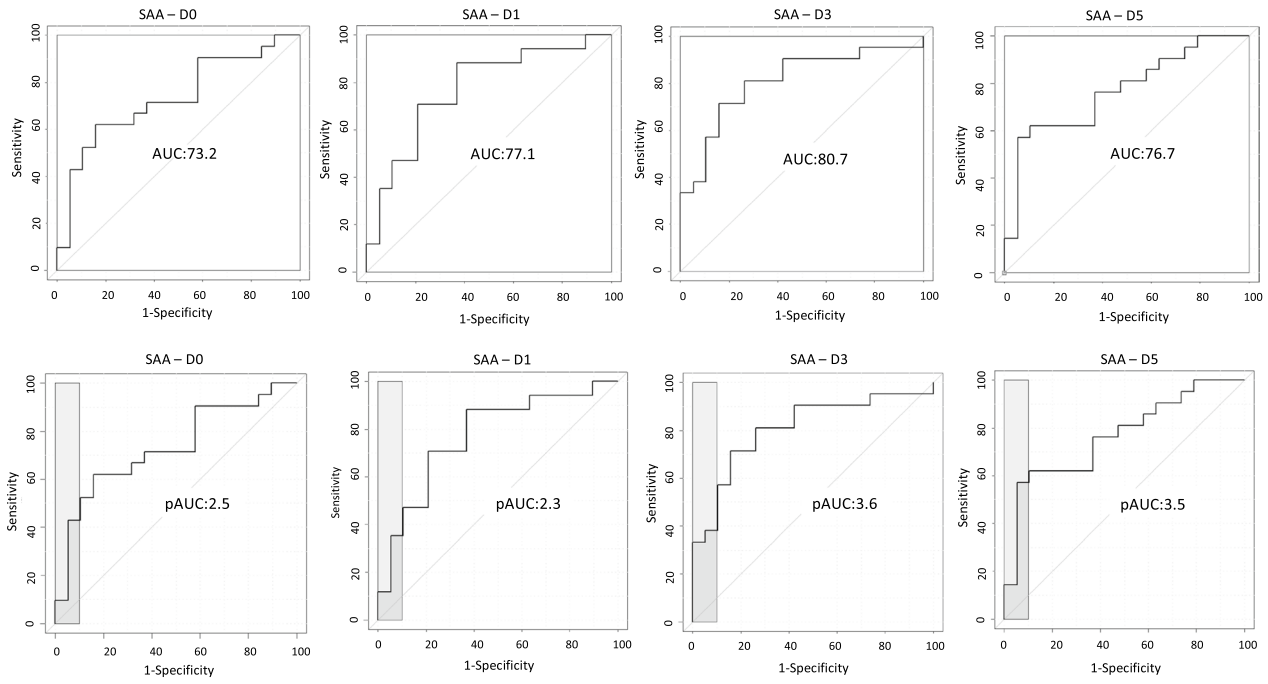

Fig. 3 ROC curves for SAA representing the ability to differentiate between patients who went on to develop an infection and those who did not, on the day of hospital admission (D0) and 1 day (D1), 3 days (D3), and 5 days after (D5) stroke onset

Table 3 Multivariate analyses of different factors predicting the presence of infection

Infection			
Predictors	**OR**	**95% CI**	***p***
SAA	3.68	(1.27–10.6)	0.047
CRP	0.37	(0.12–1.17)	0.09
WBC	1.24	(0.86–1.79)	0.24
NIHSS	1.18	(1.01–1.38)	0.031

SAA1/2 is an efficient infection-risk prediction marker in stroke patients.

SAA1/2 has already been described as a potential marker of inflammation and infection in several pathological conditions, including stroke and subarachnoid hemorrhage, [35]. In a case-controlled study involving 54 patients, levels of acute-phase proteins were significantly higher in stroke patients who developed an infection during the month preceding a cerebrovascular event than in those who did not develop one. During the month following the stroke, concentrations of SAA started being significantly higher in patients who went on to develop an infection than in those who had only had an infection preceding the cerebrovascular event at 3 days after the onset of symptoms [36]. This highlighted that the acute-phase response was clearly related to the development of infections. In another study, of 60 patients, levels of SAA were significantly higher in 45 patients with stroke than in the 15 control patients without stroke. SAA concentrations increased between days one and three in patients with a cerebral infarction complicated by an infectious inflammatory process [37]. These results suggested a correlation between the acute-phase response and the development of an infection. Nevertheless, to the best of our knowledge, until now no one had evaluated the ability of these acute-phase molecules to act as predictors of infection.

In a population of 81 subarachnoid hemorrhage patients, SAA concentrations measured at hospital admission predicted which patients would develop an infection during their hospital stay with an accuracy of 76% [38]. We therefore decided to perform the same analysis using ischemic stroke patients in the present study. As already shown, very similar results were obtained.

To the best of our knowledge, our study is the first to assess the predictive value of SAA concentrations while taking into account the time points of measurements as well as the diagnosis. We found that the SAA concentration was able to detect 42.9% of the stroke patients who had a very high certainty of going on to develop an infectious complication.

Human SAA is an acute-phase protein primarily expressed by the liver [39]. There are four different but closely related genes responsible of the protein's different isoforms. In humans, the production of SAA1 and SAA2 takes place under inflammatory conditions. SAA3 is a pseudo-gene, and SAA4 encodes a protein that is produced constitutively [40]. Inflammatory SAA1 and SAA2 share around 90% of their gene sequence. Due to the similarities between both, immunoassays have been unable to differentiate between them [39], and studies to date have been unable to determine which of the isoforms is most associated with infectious and inflammatory processes. In the present study, we used the PRM method to track each isoform and evaluate its contribution. As shown in Additional file 2: Fig. 1, both SAA1 and SAA2 are related to infection development. The FFGHGAEDSLADQAANEWGR peptide, unique to SAA1, and the tryptic SAA2 GPGGAWAAEVISNAR peptide appeared to be significantly more abundant in patients suffering from an infection than in patients without one.

SAA concentrations are most likely higher in stroke patients suffering from infections due to its role in attracting leukocytes and immune cells to the sites of tissue damage, infection, or inflammation [41, 42]. As previously described, inflammation is an important part of the reactions taking place after an ischemic event. Indeed, blood derived leukocytes and microglia will be activated from minutes to hours after a cerebrovascular event [43]. Recruitment, activation, and adhesion of leukocytes to the endothelium will happen at the same time as neutrophils and monocytes/macrophages transmigrate into the location of the cerebral infarction [44]. During this process of brain damage, the acute-phase response will also activate acute-phase proteins as SAA, CRP, haptoglobin, α1-acid glycoprotein, α1-antichymiotripsin appear increasingly in the blood [44].

The present study has certain limitations. (1) The cohort was small and its results should be validated in a larger cohort of patients in order to have sufficient samples for the subgroup analyses (infection, no-infection). (2) The study proposed SAA as a promising prognostic infection marker in stroke patients. Nevertheless, combining SAA concentrations with other clinical scales (NIHSS) or scores could improve the accuracy of the association. Different combinations should be tested to evaluate the potential added value of a panel of markers. (3) Another point which remains to be investigated is why SAA concentrations become elevated in patients developing an infection much earlier than CRP does, for example. The present study postulated that this was due to its role in inflammation, but are we thus measuring inflammation or are we facing a post-infection inflammation phenomenon? As previously reported, the acute-phase response is more prominent in patients who develop an infection during hospitalization, but

SAA concentrations were already higher when a previous bacterial infection was present. A detailed study should be performed to compare all these factors. (4) Different isoforms of SAA did not seem to act differently in conditions of inflammation. Nevertheless, the present study was only able to measure one tryptic peptide from each isoform. Further studies should target different peptides corresponding to the different isotypes of each isoform in order to perform a give a more detailed analysis of the role of SAA in infected stroke patients. Finally, to translate this study's results into clinical practice, a point of care test should be developed in order to provide results in minutes and ensure better, faster patient management.

Conclusions

In a small cohort of stroke patients, we were able to demonstrate that the concentrations of SAA1/2 measured at hospital admission could be used to predict post-stroke infection. Applying SAA measurement in clinical settings could drastically improve patient management and, consequently, their associated outcomes. Further large, multicenter validation studies are needed to confirm these results.

Additional files

Additional file 1. List of proteins identified when comparing the proteomes of five infected and five non-infected patients.

Additional file 2. Ratio of FFGHGAEDSLADQAANEWGR and GPGGAWAAEVISNAR peptide abundance between patients who went on to develop an infection and those who did not.

Additional file 3. Kinetics of SAA concentrations at hospital admission, 1 day, 3 days and 5 days after stroke onset, including discovery step patients only (a) and verification step patients only (b).

Abbreviations

AUC: area under the ROC curve; A2DS2: age, atrial fibrillation, dysphagia, sex, stroke severity; CDC: Centers for Disease Control and Prevention; CI: confidence interval; CID: collision-induced dissociation; CRP: C-reactive protein; CSF: cerebrospinal fluid; CT: computed tomography; ELISA: enzyme-linked immunosorbent assay; ECG: electrocardiogram; FDR: false discovery rate; HCD: higher-energy collisional dissociation; HPLC: high-performance liquid chromatography; HRP: horseradish peroxidase; IQR: interquartile range; IPG: immobilized pH gradient; MSD: mesoscale discovery; NIHSS: National Institutes of Health Stroke Scale; OGE: off-gel electrophoresis; OT: OrbiTrap; PRM: parallel reaction monitoring; ROC: receiver operating characteristic; PCT: procalcitonin; SAA1/2: serum amyloid 1/2; SE: sensitivity; SP: specificity; TEAB: tetraethylammonium bromide; TCEP: tris-(2-carboxyethil) phosphine hydrochloride; TMT: tandem mass tag; TOAST: trial of org 1072 in acute stroke treatment; WBC: white blood cells.

Authors' contributions

LA contributed to data acquisition and analysis as well as to drafting the manuscript. VLF contributed to data acquisition and analysis as well as to drafting the manuscript. JS contributed to sample collection and to correction and final approval of the manuscript. JM contributed to sample collection and to correction and final approval of the manuscript. MK contributed to sample collection and to correction and final approval of the manuscript. JCS contributed to data interpretation and to correction. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All the datasets included in the present manuscript are presented in the main manuscript or additional supporting files.

Ethics approval and consent to participate

We performed a post hoc analysis of a prospective cohort study including 40 ischemic stroke patients (ClinicalTrials.gov.NCT00390962) who were hospitalized consecutively at the University Hospital of Basel (Switzerland) between November 2006 and November 2007. The study was conducted according to the principles expressed in the Declaration of Helsinki, and the study protocol was approved by the local ethics committee. Before enrolment, informed consent was obtained from patients, their relatives, or their legal guardians.

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References

1. GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet 2015;385(9963):117–71.
2. Truelsen T, Piechowski-Jozwiak B, Bonita R, Mathers C, Bogousslavsky J, Boysen G. Stroke incidence and prevalence in Europe: a review of available data. Eur J Neurol. 2006;13:581–98.
3. Warlow C, Sudlow C, Dennis M, Wardlaw J, Sandercock P. Stroke. Lancet. 2003;362:1211–24.
4. Langhorne P, Stott DJ, Robertson L, MacDonald J, Jones L, McAlpine C, Dick F, Taylor GS, Murray G. Medical complications after stroke. Multicent Study. 2000;31:1223–9.
5. Tirschwell DL, Kukull WA, Longstreth WT Jr. Medical complications of ischemic stroke and length of hospital stay: experience in Seattle, Washington. J Stroke Cerebrovasc Dis. 1999;8:336–43.
6. Al-Khaled M, Matthis C, Eggers J. Predictors of in-hospital mortality and the risk of symptomatic intracerebral hemorrhage after thrombolytic therapy with recombinant tissue plasminogen activator in acute ischemic stroke. J Stroke Cerebrovasc Dis. 2014;23:7–11.
7. Hug A, Murle B, Dalpke A, Zorn M, Liesz A, Veltkamp R. Usefulness of serum procalcitonin levels for the early diagnosis of stroke-associated respiratory tract infections. Neurocrit Care. 2011;14:416–22.
8. Chamorro Á, Urra X, Planas AM. Infection after acute ischemic stroke. Manif Brain Induc Immunodepress. 2007;38:1097–103.
9. Weimar C, Roth MP, Zillessen G, Glahn J, Wimmer ML, Busse O, Haberl RL, Diener HC. Complications following acute ischemic stroke. Eur Neurol. 2002;48:133–40.
10. Westendorp WF, Nederkoorn PJ, Vermeij JD, Dijkgraaf MG, van de Beek D. Post-stroke infection: a systematic review and meta-analysis. BMC Neurol. 2011;11:110.

11. Marrie TJ, Wu L. Factors influencing in-hospital mortality in community-acquired pneumonia: a prospective study of patients not initially admitted to the ICU. Chest. 2005;127:1260–70.
12. Chamorro A, Urra X, Planas AM. Infection after acute ischemic stroke: a manifestation of brain-induced immunodepression. Stroke. 2007;38(3):1097–103.
13. Dirnagl U, Klehmet J, Braun JS, Harms H, Meisel C, Ziemssen T, Prass K, Meisel A. Stroke-induced immunodepression: experimental evidence and clinical relevance. Stroke. 2007;38:770–3.
14. Azurmendi L, Degos V, Tiberti N, Kapandji N, Sanchez-Pena P, Sarrafzadeh A, Puybasset L, Turck N, Sanchez JC. Neopterin plasma concentrations in patients with aneurysmal subarachnoid hemorrhage: correlation with infection and long-term outcome. J Neurosurg. 2016;124(5):1287–99.
15. Fluri F, Morgenthaler NG, Mueller B, Christ-Crain M, Katan M. Copeptin, procalcitonin and routine inflammatory markers-predictors of infection after stroke. PLoS ONE. 2012;7:e48309.
16. Gumbinger C, Hug A, Murle B, Berger B, Zorn M, Becker KP, Zimmermann S, Dalpke AH, Veltkamp R. Early blood-based microbiological testing is ineffective in severe stroke patients. J Neurol Sci. 2013;325:46–50.
17. Hoffmann S, Malzahn U, Harms H, Koennecke HC, Berger K, Kalic M, Walter G, Meisel A, Heuschmann PU. Development of a clinical score (A2DS2) to predict pneumonia in acute ischemic stroke. Stroke. 2012;43:2617–23.
18. Smith CJ, Bray BD, Hoffman A, Meisel A, Heuschmann PU, Wolfe CD, Tyrrell PJ, Rudd AG. Can a novel clinical risk score improve pneumonia prediction in acute stroke care? A UK multicenter cohort study. J Am Heart Assoc. 2015;4:e001307.
19. Ji R, Shen H, Pan Y, Wang P, Liu G, Wang Y, Li H, Wang Y. Novel risk score to predict pneumonia after acute ischemic stroke. Stroke. 2013;44:1303–9.
20. Finlayson O, Kapral M, Hall R, Asllani E, Selchen D, Saposnik G. Risk factors, inpatient care, and outcomes of pneumonia after ischemic stroke. Neurology. 2011;77:1338–45.
21. Garner JS, Jarvis WR, Emori TG, Horan TC, Hughes JM. CDC definitions for nosocomial infections. Am J Infect Control. 1988;16:128–40.
22. Adams HP, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in acute stroke treatment. Stroke. 1993;24(1):35–41.
23. Dayon L, Turck N, Kienle S, Schulz-Knappe P, Hochstrasser DF, Scherl A, Sanchez JC. Isobaric tagging-based selection and quantitation of cerebrospinal fluid tryptic peptides with reporter calibration curves. Anal Chem. 2010;82:848–58.
24. Dayon L, Turck N, Scherl A, Hochstrasser DF, Burkhard PR, Sanchez J-C. From relative to absolute quantification of tryptic peptides with tandem mass tags: application to cerebrospinal fluid. CHIMIA Int J Chem. 2010;64:132–5.
25. Dayon L, Pasquarello C, Hoogland C, Sanchez J-C, Scherl A. Combining low- and high-energy tandem mass spectra for optimized peptide quantification with isobaric tags. J Proteomics. 2010;73:769–77.
26. Gluck F, Hoogland C, Antinori P, Robin X, Nikitin F, Zufferey A, Pasquarello C, Fétaud V, Dayon L, Müller M, et al. EasyProt—an easy-to-use graphical platform for proteomics data analysis. J Proteomics. 2013;79:146–60.
27. Elias JE, Gygi SP. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods. 2007;4:207–14.
28. Breitwieser FP, Muller A, Dayon L, Kocher T, Hainard A, Pichler P, Schmidt-Erfurth U, Superti-Furga G, Sanchez JC, Mechtler K, et al. General statistical modeling of data from protein relative expression isobaric tags. J Proteome Res. 2011;10:2758–66.
29. Tiberti N, Hainard A, Lejon V, Robin X, Ngoyi DM, Turck N, Matovu E, Enyaru J, Ndung'u JM, Scherl A, et al. Discovery and verification of osteopontin and beta-2-microglobulin as promising markers for staging human African trypanosomiasis. Mol Cell Proteomics MCP. 2010;9:2783–95.
30. Tan HT, Tan S, Lin Q, Lim TK, Hew CL, Chung MC. Quantitative and temporal proteome analysis of butyrate-treated colorectal cancer cells. Mol Cell Proteomics. 2008;7:1174–85.
31. Salvisberg C, Tajouri N, Hainard A, Burkhard PR, Lalive PH, Turck N. Exploring the human tear fluid: discovery of new biomarkers in multiple sclerosis. Proteomics Clin Appl. 2014;8:185–94.
32. Kim YJ, Gallien S, El-Khoury V, Goswami P, Sertamo K, Schlesser M, Berchem G, Domon B. Quantification of SAA1 and SAA2 in lung cancer plasma using the isotype-specific PRM assays. Proteomics. 2015;15:3116–25.
33. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, Kern R, Tabb DL, Liebler DC, MacCoss MJ. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics. 2010;26:966–8.
34. Garcia-Berrocoso T, Penalba A, Boada C, Giralt D, Cuadrado E, Colome N, Dayon L, Canals F, Sanchez JC, Rosell A, Montaner J. From brain to blood: new biomarkers for ischemic stroke prognosis. J Proteomics. 2013;94:138–48.
35. Azurmendi L, Degos V, Tiberti N, Kapandji N, Sanchez P, Sarrafzadeh A, Puybasset L, Turck N, Sanchez J-C. Measuring serum amyloid A for infection prediction in aneurysmal subarachnoid hemorrhage. J Proteome Res. 2015;14:3948–56.
36. Syrjanen J, Teppo AM, Valtonen VV, Iivanainen M, Maury CP. Acute phase response in cerebral infarction. J Clin Pathol. 1989;42:63–8.
37. Ilzecka J, Stelmasiak Z. Prognostic importance of monitoring serum amyloid A protein (SAA) in patients with cerebral infarction. Acta Clin Croat. 2000;39:139–46.
38. Azurmendi L, Degos V, Tiberti N, Kapandji N, Sanchez P, Sarrafzadeh A, Puybasset L, Turck N, Sanchez JC. Measuring serum amyloid A for infection prediction in aneurysmal subarachnoid hemorrhage. J Proteome Res. 2015;14:3948–56.
39. Uhlar CM, Whitehead AS. serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem. 1999;265:501–23.
40. Malle EDBF. Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice. Eur J Clin Invest. 1996;26:427–35.
41. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999;340:448–54.
42. Nakayama T, Sonoda S, Urano T, Yamada T, Okada M. Monitoring both serum amyloid protein A and C-reactive protein as inflammatory markers in infectious diseases. Clin Chem. 1993;39:293–7.
43. del Zoppo GJ, Becker KJ, Hallenbeck JM. Inflammation after stroke: Is it harmful? Arch Neurol. 2001;58:669–72.
44. Ahmad M, Graham SH. Inflammation after stroke: mechanisms and therapeutic approaches. Transl Stroke Res. 2010;1:74–84.

Chapter VII

Discussion, perspectives and conclusions

The improvement of outcome and management of patients suffering from stroke has been an important challenge since many years(20). Excluding the high mortality produced as a consequence of the initial cerebrovascular accident, common complications occurring after the symptom onset are the main cause of outcome worsening and death (155, 196, 222).

Post-stroke infections are one of the most common complications developing during the hospital stay and leading to high rates of morbidity/mortality (191, 201, 223). Early initiation of antibiotherapy is recommended once the infection has been diagnosed (97). However, at present, infection diagnosis is a challenging task for physicians as the proposed criteria in the different studies are inconsistent. The current clinical practice based on the performance of blood cultures is time consuming and low, which highlights the need of better diagnostic methods including biomarkers able to identify post-stroke infections in a more accurate and earlier way (94). Several studies were performed to discover biomarkers able to detect these complications before its development in order to apply the necessary treatment to reduce their impact. However, for instance there is a lack of a gold standard.

1. Main results of this thesis project

1.1. Infection prediction in aSAH patients

The first objective of this thesis project was to find a biomarker able to predict aSAH patients that will develop an infection during their hospital stay. For this purpose, we applied the classical strategies of biomarker discovery, verification and validation steps explained during the introduction of this manuscript. For the discovery step, knowledge obtained from the literature and untargeted proteomic methods were applied. For the verification and validation steps of the most interesting molecules, immunoassays and targeted proteomics were applied. A cohort of 198 aSAH patients collected from Pitié-Salpêtrière Hospital of Paris was used for verification and an additional population of 63 aSAH patients coming from Charité Universitätsmedizin Hospital in Berlin was used for the validation of the results.

According to the literature, inflammation and immune responses are the key elements in the pathogenesis of this disease, as well as in the development of infectious complications occurring after the symptom onset. The immune system takes a key role in the brain damages produced by the initial accident, and similarly, the injured brain, produces an immunosuppressive reaction that promotes the development of infections; more concretely T cells and natural killer (NK) cells suppress the production of interferon gamma (IFN-γ), altering importantly the antibacterial defense. Following this theory, we first evaluated the capacity of neopterin to act as an infection predictor in patients suffering from aSAH. Neopterin, is a metabolite produced by monocytes-

macrophages, which provides information about the TH1 response activity and about the activity of the cellular immune system. It has been used in the past as a biomarker in several inflammatory and infectious diseases (185, 224, 225). Furthermore, in patients with cerebrovascular accidents it has been shown to have significantly higher concentrations than in control ones and to correlate with the long term outcome of these patients (187, 226).

To increase the set of potential biomarkers for infection prediction, we did not limit our research to bibliography and performed quantitative mass spectrometry (TMT-6 plex) approach, to compare the proteome of infected and non-infected aSAH patients at hospital admission. Among the 17 significantly regulated proteins between the two groups of patients, serum amyloid A, appeared to have the higher ratio when comparing the two groups of patients. The liver secretes this acute phase protein during inflammation. In patients with acute ischemic stroke it has been shown to be a useful marker for the diagnosis of atherotrombotic stroke as well as to act as a prognostic marker (86). However, neither SAA nor neopterin have been evaluated so far as potential infection markers in aSAH patients.

In the present thesis project we found that both candidates, showed promising capacity to act as infection markers: neopterin concentrations appeared to well correlate with infection development from three days after hospital admission and onwards (Chapter III). Similarly, concentrations of SAA appeared to be significantly increased in aSAH patients developing and infection than in non-infected ones (Chapter iV). Furthermore, in this case, significant differences were already found at hospital admission, which highlights SAA as an earlier marker than neopterin and a really promising tool to use in clinical practice as an infection risk stratificator. Even if the accuracy values of SAA (at hospital admission: 75.3%) and NP (three days after hospital admission: 68.5%) were quite promising, it has previously been shown in stroke patients developing infectious complications, that the combination of individual markers with clinical parameters could importantly improve the prediction accuracy of single markers. The A2DS2 score, with an accuracy of 83.6% has become one of the latest promising tools to predict post-stroke associated pneumonia. In this thesis project, we tested the hypothesis that the combination of SAA or neopterin with other biomarkers or clinical parameters could improve the individual accuracy of each molecule. We used our home-made Panelomix tool to find the best combination of markers. To select the individual parameters that could be introduced in the panel, we combined the two potential biomarkers with already described infection risk factors: clinical scales as WFNS or Fisher, the age of the patients and the values of WBC at hospital admission. As previously shown in chapter IV, the combination formed by SAA, WFNS, WBC and age increased importantly the total values of SP and SE when comparing with individual markers.

For a fixed SP of 95-100%, SAA and NP obtained SE values of 26.56 and 10.14 respectively. The panel was able to importantly increase this sensitivity to reach a value of 100% SP for 64.3% SE (Chapter V).

1.2. **Infection prediction in ischemic stroke patients**

The second objective of this thesis project was to find a biomarker for infection prediction in ischemic stroke patients. Even if this is a relatively new research approach when comparing with diagnosis or stroke prognosis research, some discovery has already been performed. One of the most studied and accepted markers until present is the CRP, an acute phase inflammatory molecule, highly related with infection and inflammation (97, 162). However, in ischemic stroke patients, it has only shown a moderate predictive value when comparing with clinical information (227). Copeptin and procalcitonin, the other two widely studied markers, have not shown the expected results. Even if they are independently associated with the development of infections, they are not early enough to administrate the antibiotic treatment in an earlier stage(97). Consequently, new biomarkers are needed to improve the management of ischemic stroke patients and avoid infection development.

For this purpose, the same workflow of discovery, verification and validation steps followed in the discovery of infection markers for aSAH patients was applied. First of all, for the discovery phase, we performed a TMT-10 plex in which we compared the plasma samples collected at hospital admission of stroke patients developing an in-hospital infection and stroke patients not developing it. Among the quantified proteins, the well already known SAA appeared to be again the only significantly regulated protein between the two groups of patients, being 2.2 times more abundant in infected patients than in non-infected ones.

For the verification of these results, plasma samples of 40 ischemic stroke patients coming from the University Hospital of Basel (Switzerland) were used. As shown in chapter VI, SAA levels appeared to be significantly higher in patients that will develop a post-stroke infection than in those without it. With an accuracy of 73.2% at hospital admission, these results confirmed that SAA could be also an interesting marker in stroke patients (Chapter VI).

Afterwards, in an attempt to validate these results, during the last phase of this thesis we measured the concentrations of SAA in 243 additional ischemic stroke patients coming from the University Hospital of Basel (Switzerland).

According to the demographic characteristics of the population, we saw that most of the patients were men (59.3%) and that the median age (IQR) of the population was 75.3 years (62.5-82). After performing univariate analysis very few parameters appeared to affect the

development of infection. Patients presenting an infection during hospitalization developed hypertension and atrial fibrillation significantly more currently than patients without infection. Similarly, patients with large vessel lesions were more prone to develop infections than those with small lesions. Finally, laboratory values at admission (SAA, CRP, PCT, WBC) and clinical scales appeared also to have an important role in infection development; more concretely, NIHSS values were significantly higher in those patients developing an infection than in those patients without it.

Following the same trend, levels of SAA (Figure 1), PCT, CRP and WBC measured at hospital admission in 59 infected and 224 non-infected patients appeared to be statistically increased in those patients that will develop an infection, postulating that they could be promising infection diagnostic tools.

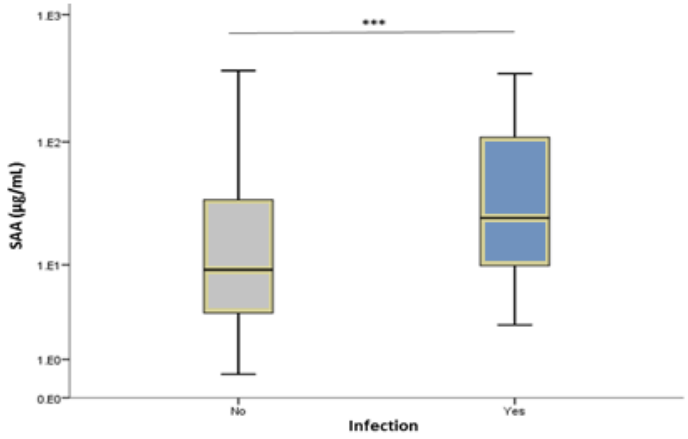

Figure 1: SAA plasma concentrations of 59 infected and 224 non-infected patients at hospital admission. Comparisons between the two groups were made using the Mann Whitney U test. *= significance level reported after the Bonferroni correction (p<0.05)

Consequently, multivariate analyses were performed to assess the association between variables. The presence of infection was set as the dependent variable and the following significantly regulated parameters (gender, NIHSS, WBC, CRP, PCT and SAA) as confounders. As represented in Table 2 only SAA displayed a relationship with the development of infections, which confirms and validate the capacity of SAA to act as an infection marker in stroke patients.

Infection					
Predictors	**OR**	**95% CI**			***P***
Gender	1.58	0.8	–	3.14	0.19
NIHSS	1.06	1.01	–	1.1	0.01
WBC	1.17	1.05	–	1.31	0.006
Log_CRP	0.9	0.57	–	1.43	0.66
Log_SAA	1.32	0.99	–	1.76	**0.053**

Table 2: multivariate logistic regression analysis with selected variables significant in univariate logistic analysis (p<0.01)

Further analyses are needed and will be soon performed to evaluate if the individual capacity of SAA could be improved by combining it with clinical scales such as NIHSS or clinical parameters. These exciting results will be included in a manuscript to be submitted to *Stroke*.

In any case, all the results produced during the attempt to find ischemic stroke and SAH infection biomarkers, have highlighted that the translational strategies were efficient for the discovery phase. SAA appears as a promising tool to classify patients at high risk of post-stroke infections reducing the risk of infection development and increasing potentially the efficacy of antibiotherapy.

2. Perspectives

One the important perspective of this thesis project is to translate the SAA into the clinical practice. However, before this important step is achieved, several issues should be solved.

2.1. **POCT development**

The development of a rapid Point-Of-Care-Test (POCT) is the last necessary step to use this biomarker at the bedside as an infection risk stratificator in patients with cerebrovascular events. For this purpose, we should first of all compare the accuracy of our already known SAA ELISA test (Mesoscale) with that of two already developed POCTS: Upper Biotech (China) for the human form and Accuplex (Ireland), a POCT used to measure the degree on inflammation and infection in race horses.

The limits of detection and quantification as well as the overall precision should be tested. Afterwards, verification should be performed using the plasma samples of 20 stroke patients coming from Zurich (10 infected and 10 non-infected) that should be used to compare the two POCTs at two different time points. Finally, in order to validate the results, SAA levels of 100 patients (50 infected and 50 non-infected) coming from Zurich and Barcelona should be tested at different time points. ROC curves will establish the discriminatory capacity of these tests to act as infection markers.

2.2. **Study of different SAA isoforms**

Apart from the practical utility of SAA, before its translation into the clinical practice, it would be also important and interesting to well identify the mechanism and the isoforms of the SAA that are related with infection development. Very interesting results obtained during this thesis

project were the observation by PRM of the different SAA isoforms (Chapter VI). As already described

during the introduction of the manuscript, SAA1 and SAA2, the two acute phase isoforms, consist in 104 amino acid residues that share around 93% of their sequence (228). Each of them present specific alleles with different frequencies: SAA1α (31%), β (32%), γ (37%) and SAA2α (65-80%) and β (20-35%) (179, 218). However, for instance, no studies have been performed to evaluate if one of the isoforms has a better and earlier performance to the detection of the infection than the others.

In the present project, levels of SAA were measured using immunoassays, which did not allow distinguishing between the SAA1 and SAA2 due to their sequence similarity. Therefore, in order to evaluate if any of the SAA isoforms had a better predictive power than the other, targeted proteomic analysis were performed. Parallel reaction monitoring (PRM) was used to accurately quantify specific peptides in complex samples (212). Preliminary results (chapter VI) performed in stroke patients with specific peptides of the total SAA (SFFSFLGEAFDGAR), SAA1 (FFGHGAEDSLADQAANEWGR) and SAA2 (GPGGAWAAEVISNAR) have not shown differences in their capacity to act as infection stratificators, finding that all the three tryptic peptides were significantly more abundant in infected patients than in non-infected ones. However, to accurately quantify the role of each isoform, absolute quantification is needed at different time points (0, 12, 24, 48 and 72h) and in larger multicentric cohorts. For this purpose, isotopically spiked synthetic peptides will be used in the near future.

2.3. **Translation of SAA into the clinical practice**

Once the knowledge of the different SAA isoforms has been figure out and the POCT has been developed, prospective studies could be launched. First of all, an observational prospective study should be performed to evaluate if the levels of SAA measured with the POCT correlate with infection such in a good way as it was obtained during the retrospective study.

In a second step, an interventional prospective study should be performed. First of all and in order to obtain accurate results with sufficient statistical power, the sample size of the study must be calculated. Estimating a power of 99% at least 27 infected patients should be included, so for a calculated prevalence of infection of 10%, a total of 270 patients (27 infected and 135 non-infected) should be required in the present study. Afterwards, patients with values of SAA above the previous established cut-off, should receive the antibiotherapy in the shortest delay.

A similar clinical trial has already been performed with procalcitonin, which postulates that the use of accurate biomarkers is the best option to guide the administration of antibiotic therapy

and to improve the associated outcome (158). The next and final step should be to evaluate the circumstances under which SAA is effective for reducing post-stroke infections and improve outcome.

2.4. **Other molecules as infection markers**

To increase the knowledge of the disease, we also preliminary investigated biomarkers that have already been related with the physiopathology of the disease. As already explained during the introduction of this manuscript, different types of cytokines, chemokines, adhesion molecules, cellular components and acute phase molecules are involved in the inflammation pathway and pathogenesis of cerebrovascular accidents (75, 78, 221, 229-231). Consequently, we selected some of the most representative molecules of each of these groups to obtain an overall image of their performance in predicting infectious complications.

In stroke patients, cytokine concentrations increase their concentrations during the first 24 hours after the brain injury and correlate with poor outcome at one year (75, 232). During this thesis project, we evaluated at hospital admission, the levels of IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL12p70, IL-13, TNF-α and IP-10 in 44 infected and 22 non-infected aSAH patients. Chemokine concentrations of MCP-1 or MIP-1α are also supposed to be significantly increased in those patients presenting with an ischemic stroke or subarachnoid hemorrhage than in control ones(78). Furthermore, their inhibition is supposed to revert the damage level. Consequently, we measured at hospital admission the concentrations of these two molecules in the same infected and non-infected patients than above. Adhesion molecules, the group of molecules that participates in inflammatory endothelial activation after a brain injury, were shown to be increased in patients with ischemic stroke or in aSAH patients presenting ischemic lesions. ICAM and VCAM, are the two most reported ones (75, 230, 233) and the two selected for the analysis in our population. Finally, we measured the levels of the two most studied biomarkers related with infection detection: WBC and CRP. Leukocyte levels are widely used to evaluate the presence of an infection in different types of diseases. However, in aSAH patients some controversy has been found as the increase in their levels produced by the impact of the initial hemorrhage did not allow predicting the patients that develop an infection from those without infection. Elevated CRP concentrations have been also widely related with infection development. In stroke patients higher concentrations have been related to poor outcome and mortality as well as to the occurrence of post-stroke infections (227). In the present project we evaluated also their concentrations in 44 infected and 22 non-infected patients.

Among all the evaluated molecules, at hospital admission MCP-1, CRP and WBC showed the most promising accuracies, reaching similar values to those obtained by SAA. Even if at this early time point cytokines did not seem to be very accurate in stratification of patients at higher risk of infection development, we can observe that their accuracies importantly increased during the hospitalization stay, obtaining very accurate values already three days after hospital admission.

Molecule	**AUC (%)** D1	***p***	**AUC (%)** D3	***p***	**AUC (%)** D5	***p***	**AUC (%)** D7	***p***	**AUC (%)** D9	***p***
Cytokines										
IFN-γ	50.2	n.s	73.5	n.s	54	n.s	61.9	n.s	54.2	n.s
IL1β	53.6	n.s	47.6	n.s	57.9	n.s	53.5	n.s	49.9	n.s
IL-2	61.5	n.s	64	n.s	63.4	n.s	51.6	n.s	57.2	n.s
IL-4	61.2	n.s	61.6	n.s	55	n.s	64.5	**0.042**	53.9	n.s
IL-6	57.9	n.s	81	**≤0.001**	80.9	**≤0.001**	75.4	**≤0.001**	74.2	**≤0.001**
IL-8	52.7	n.s	54.6	n.s	58.2	n.s	61.2	n.s	57.5	n.s
IL-10	49.8	n.s	75.8	**≤0.001**	72.4	n.s	59.5	n.s	64.8	**0.028**
IL12p70	54.1	n.s	51.1	n.s	50	n.s	57	n.s	50.9	n.s
IL-13	62.2	n.s	61.3	n.s	52.7	n.s	60.5	n.s	51.2	n.s
TNF-α	56.9	n.s	71.7	**0.002**	72.1	**0.001**	68	**0.009**	58.6	n.s
IP-10	53.5	n.s	64.1	**0.043**	54	n.s	62.1	n.s	50.2	n.s
Chemokines										
MCP-1	73.2	**0.005**								
MPI-1α	61.5	n.s								
Adhesion molecules										
ICAM	69	**0.02**	64.4	0.03	70.9	0.002	71.2	0.002	70.3	0.004
VCAM	65.6	n.s	65	0.03	72.2	0.001	65.5	0.026	64.5	0.04
Cellular components										
WBC	76.3	**0.008**	73.6	**0.007**	57.7	n.s	54.8	n.s	62.7	n.s
Acute phase proteins										
CRP	73.8	**0.009**	73.8	**0.001**	85	**≤0.001**	84.4	**≤0.001**	78.6	**≤0.001**
SAA	76	**≤0.001**	69.5	**0.005**	79.4	**≤0.001**	81.7	**≤0.001**	79.2	**≤0.001**
Metabolites										
NP	58.5	n.s	68.4	**0.008**	81.6	**≤0.001**	82.1	**≤0.001**	81.1	**≤0.001**

Table 3: accuracy of different molecules to differentiate between patients that will and will not develop an infection during their hospital stay.

These promising preliminary results performed in the cohort of aSAH patients coming from Paris, should be validated in the available aSAH cohort of patients coming from Berlin. The combination of the most accurate molecules with other markers or clinical parameters should be performed to evaluate the improvement on the individual accuracy. Finally, pathway analysis should be suitable to better understand the role of the different molecules in the pathophysiology of the disease. Each of these inflammatory molecules, have been described individually as having different performances in stroke patients. Present results could be used to support this theory and to get new insights in the disease.

2.5. **Delayed cerebral ischemia (DCI) biomarker discovery**

The main goal of this thesis project was focused on the discovery of biomarkers for the prediction of patients at risk of infection development. However, in aSAH one of the most feared complications is vasospasm and DCI. Many studies were performed to understand the mechanisms responsible of its development as well as to try to find new alternative treatments (53, 57, 74, 222, 234, 235). However, until present no conclusive findings have been found.

We hypothesized, that literature and proteomics analysis could help to increase the knowledge of this deleterious complication. Consequently, using proteomics tools we compared first of all, the proteins coming from patients with and without DCI, to find biomarkers able to avoid the development of vasospasm and find predictor biomarkers. The same workflow followed for the discovery of infection markers was also used in the discovery step associated with DCI: we set up a TMT-10 plex quantitative MS to analyze plasma samples obtained at the day of vasospasm onset, two days before the symptom onset and the equivalent day in patients without vasospasm.

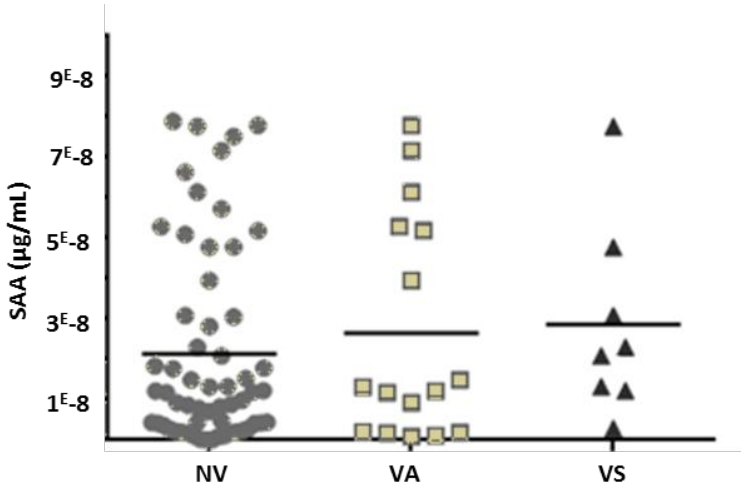

Figure 3: concentrations of SAA in patients without vasospasm (NV), in patients with angiographic vasospasm (VA) and in patients with symptomatic vasospasm (VS).

When comparing the vasospasm patients two days before the symptom onset with no-vasospasm patients, 11 proteins were identified as significantly regulated between the two groups of patients. Among them, neuromodulin and SAA, showed the highest ratios. A first attempt to verify neuromodulin results was performed, but the low sensitivity and reproducibility of the commercialized immunoassays, did not allow obtaining a conclusive results.

Levels of SAA, measured with the already known Mesoscale assay, appeared to be slightly higher in patients with symptomatic vasospasm (n= 8) and angiographic vasospasm (n=16) than in

those without vasospasm (n=53) (Figure 2). Even if the differences did not appear to be significant, further verification in higher number of patients should be performed for SAA and other potentially interesting proteins.

2.6. **Inflammation in vasospasm pathogenesis**

Apart from SAA and considering previous literature search, we decided to follow the hypothesis that inflammation is one of the most promising ways to find new treatments and understand the vasospasm development. Proinflammatory cytokines such as IL-1β, IL-6, IL-8 and TNF-α have been shown to be elevated in patients with vasospasm (236-240). Furthermore, IL-6 levels increase importantly just after aSAH, postulating that it could be an early marker able to predict vasospasm development (239, 240).

Consequently, in our cohort of patients coming from Paris, we compared the levels of TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL12p70, IL-13, IP-10 in patients with and without vasospasm at different time points: two days before the vasospasm onset (V-2), one day before (V-1), the day of vasospasm (V) and one and two days after (V+1 and V+2). No significant differences were found between the different groups. The low number of patients suffering from angiographic and symptomatic vasospasm made difficult to obtain significant differences among groups.

New cohorts with more clear and detailed definitions of vasospasm, DCI, angiographic and symptomatic vasospasm are needed to be able to correctly evaluate the impact of these complications in patient's management and outcome. A new cohort of 63 aSAH patients coming from Charité Universitätsmedizin Hospital in Berlin is available for future analysis.

3. Conclusions

Post-stroke infections are one of the most important causes of outcome worsening and death in patients that survive to the initial cerebrovascular accidents. Early identification of patients at risk of infection development is crucial to administrate the antibiotherapy in the shortest delay and to improve their associated outcome. However, due to the low specificity of the symptoms, diagnosis step has become an important challenge for physicians, highlighting the need of early infection prognostic biomarkers. In the present thesis project, we demonstrated that SAA could be a promising marker to predict aSAH and ischemic patients susceptible to develop in-hospital infections. In combination with other markers it is able to predict at hospital admission 60% of patients that will develop an infection, becoming consequently an excellent tool to introduce the

antibiotherapy in an earlier stage, to better manage the patients and to improve their associated outcomes.

The development of a SAA POCT is a crucial step to translate this marker to the bedside, to improve the quality of life of the patients and to decrease health care costs.

4. References

1. Feigin, V. L.; Forouzanfar, M. H.; Krishnamurthi, R.; Mensah, G. A.; Connor, M.; Bennett, D. A.; Moran, A. E.; Sacco, R. L.; Anderson, L.; Truelsen, T.; O'Donnell, M.; Venketasubramanian, N.; Barker-Collo, S.; Lawes, C. M.; Wang, W.; Shinohara, Y.; Witt, E.; Ezzati, M.; Naghavi, M.; Murray, C., Global and regional burden of stroke during 1990-2010: findings from the Global Burden of Disease Study 2010. Lancet 2014, 383, (9913), 245-54.

2. Nagata, K.; Suzuki, K., [Update on stroke epidemiology]. Brain Nerve 2013, 65, (7), 857-70.

3. Donnan, G. A.; Fisher, M.; Macleod, M.; Davis, S. M., Stroke. The Lancet 2008, 371, (9624), 1612-1623.

4. Li, J.; Wang, D.; Tao, W.; Dong, W.; Zhang, J.; Yang, J.; Liu, M., Early consciousness disorder in acute ischemic stroke: incidence, risk factors and outcome. BMC Neurol 2016, 16, (1), 140.

5. Howard, V. J., Reasons Underlying Racial Differences in Stroke Incidence and Mortality. Stroke; a journal of cerebral circulation 2013, 44, (6 0 1), S126-S128.

6. Brønnum-Hansen, H.; Davidsen, M.; Thorvaldsen, P., Long-Term Survival and Causes of Death After Stroke. Stroke 2001, 32, (9), 2131-2136.

7. Easton, J. D.; Saver, J. L.; Albers, G. W.; Alberts, M. J.; Chaturvedi, S.; Feldmann, E.; Hatsukami, T. S.; Higashida, R. T.; Johnston, S. C.; Kidwell, C. S.; Lutsep, H. L.; Miller, E.; Sacco, R. L., Definition and Evaluation of Transient Ischemic Attack. A Scientific Statement for Healthcare Professionals From the American Heart Association/American Stroke Association Stroke Council; Council on Cardiovascular Surgery and Anesthesia; Council on Cardiovascular Radiology and Intervention; Council on Cardiovascular Nursing; and the Interdisciplinary Council on Peripheral Vascular Disease: The American Academy of Neurology affirms the value of this statement as an educational tool for neurologists. 2009, 40, (6), 2276-2293.

8. van der Worp , H. B.; van Gijn , J., Acute Ischemic Stroke. New England Journal of Medicine 2007, 357, (6), 572-579.

9. Thomson, R., Evidence based implementation of complex interventions. BMJ 2009, 339.

10. Whisnant, J. P., Effectiveness versus efficacy of treatment of hypertension for stroke prevention. Neurology 1996, 46, (2), 301-307.

11. Reynolds, K.; Lewis, B.; Nolen, J. L.; Kinney, G. L.; Sathya, B.; He, J., Alcohol consumption and risk of stroke: A meta-analysis. JAMA 2003, 289, (5), 579-588.

12. Group, U. K. P. D. S., Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). The Lancet 1998, 352, (9131), 837-853.

13. Adams, H. P.; Bendixen, B. H.; Kappelle, L. J.; Biller, J.; Love, B. B.; Gordon, D. L., Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in Acute Stroke Treatment. Stroke. 1993, 24.

14. Lavallee, P.; Amarenco, P., Stroke subtypes and interventional studies for transient ischemic attack. Front Neurol Neurosci 2014, 33, 135-46.

15. Furie , B.; Furie , B. C., Mechanisms of Thrombus Formation. New England Journal of Medicine 2008, 359, (9), 938-949.

16. Kisialiou, A.; Grella, R.; Carrizzo, A.; Pelone, G.; Bartolo, M.; Zucchella, C.; Rozza, F.; Grillea, G.; Colonnese, C.; Formisano, L.; Lembo, M.; Puca, A. A.; Vecchione, C., Risk factors and acute ischemic stroke subtypes. Journal of the Neurological Sciences 2014, 339, (1–2), 41-46.

17. Singer, O. C.; Humpich, M. C.; Laufs, H.; Lanfermann, H.; Steinmetz, H.; Neumann-Haefelin, T., Conjugate Eye Deviation in Acute Stroke. Incidence, Hemispheric Asymmetry, and Lesion Pattern 2006, 37, (11), 2726-2732.

18. Muir, K. W.; Weir, C. J.; Murray, G. D.; Povey, C.; Lees, K. R., Comparison of Neurological Scales and Scoring Systems for Acute Stroke Prognosis. Stroke 1996, 27, (10), 1817-1820.

19. Wilson, J. T. L.; Hareendran, A.; Grant, M.; Baird, T.; Schulz, U. G. R.; Muir, K. W.; Bone, I., Improving the Assessment of Outcomes in Stroke. Use of a Structured Interview to Assign Grades on the Modified Rankin Scale 2002, 33, (9), 2243-2246.

20. Jauch, E. C.; Saver, J. L.; Adams, H. P., Jr.; Bruno, A.; Connors, J. J.; Demaerschalk, B. M.; Khatri, P.; McMullan, P. W., Jr.; Qureshi, A. I.; Rosenfield, K.; Scott, P. A.; Summers, D. R.; Wang, D. Z.; Wintermark, M.; Yonas, H., Guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke 2013, 44, (3), 870-947.

21. McLaughlin, P. D.; Moloney, F.; O'Neill, S. B.; James, K.; Crush, L.; Flanagan, O.; Maher, M. M.; Wyse, G.; Fanning, N., CT of the head for acute stroke: Diagnostic performance of a tablet computer prior to intravenous thrombolysis. J Med Imaging Radiat Oncol 2017.

22. Kidwell, C. S.; Chalela, J. A.; Saver, J. L.; et al., Comparison of mri and ct for detection of acute intracerebral hemorrhage. JAMA 2004, 292, (15), 1823-1830.

23. Bederson, J. B.; Connolly, E. S.; Batjer, H. H.; Dacey, R. G.; Dion, J. E.; Diringer, M. N.; Duldner, J. E.; Harbaugh, R. E.; Patel, A. B.; Rosenwasser, R. H., Guidelines for the Management of Aneurysmal Subarachnoid Hemorrhage: A Statement for Healthcare Professionals From a

Special Writing Group of the Stroke Council, American Heart Association. Stroke 2009, 40, (3), 994-1025.

24. Southerland, A. M., Clinical Evaluation of the Patient With Acute Stroke. Continuum (Minneap Minn) 2017, 23, (1, Cerebrovascular Disease), 40-61.

25. Emberson, J.; Lees, K. R.; Lyden, P.; Blackwell, L.; Albers, G.; Bluhmki, E.; Brott, T.; Cohen, G.; Davis, S.; Donnan, G.; Grotta, J.; Howard, G.; Kaste, M.; Koga, M.; von Kummer, R.; Lansberg, M.; Lindley, R. I.; Murray, G.; Olivot, J. M.; Parsons, M.; Tilley, B.; Toni, D.; Toyoda, K.; Wahlgren, N.; Wardlaw, J.; Whiteley, W.; del Zoppo, G. J.; Baigent, C.; Sandercock, P.; Hacke, W., Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. The Lancet 384, (9958), 1929-1935.

26. Roth, J. M., Recombinant tissue plasminogen activator for the treatment of acute ischemic stroke. Proceedings (Baylor University. Medical Center) 2011, 24, (3), 257-259.

27. Scott Burgin, W.; Alexandrov, A. V., Deterioration following improvement with tPA therapy: Carotid thrombosis and reocclusion. Neurology 2001, 56, (4), 568-570.

28. Dubinsky, R.; Lai, S.-M., Mortality of stroke patients treated with thrombolysis: Analysis of nationwide inpatient sample. Neurology 2006, 66, (11), 1742-1744.

29. Alper, B. S.; Malone-Moses, M.; McLellan, J. S.; Prasad, K.; Manheimer, E., Thrombolysis in acute ischaemic stroke: time for a rethink? BMJ : British Medical Journal 2015, 350.

30. Caceres, J. A.; Goldstein, J. N., Intracranial Hemorrhage. Emergency medicine clinics of North America 2012, 30, (3), 771-794.

31. Hemphill, J. C.; Greenberg, S. M.; Anderson, C. S.; Becker, K.; Bendok, B. R.; Cushman, M.; Fung, G. L.; Goldstein, J. N.; Macdonald, R. L.; Mitchell, P. H.; Scott, P. A.; Selim, M. H.; Woo, D., Guidelines for the Management of Spontaneous Intracerebral Hemorrhage. A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association 2015, 46, (7), 2032-2060.

32. Feldmann, E.; Broderick, J. P.; Kernan, W. N.; Viscoli, C. M.; Brass, L. M.; Brott, T.; Morgenstern, L. B.; Wilterdink, J. L.; Horwitz, R. I., Major Risk Factors for Intracerebral Hemorrhage in the Young Are Modifiable. Stroke 2005, 36, (9), 1881-1885.

33. Feigin, V. L.; Rinkel, G. J. E.; Lawes, C. M. M.; Algra, A.; Bennett, D. A.; van Gijn, J.; Anderson, C. S., Risk Factors for Subarachnoid Hemorrhage. An Updated Systematic Review of Epidemiological Studies 2005, 36, (12), 2773-2780.

34. McDonald, R. J.; McDonald, J. S.; Bida, J. P.; Kallmes, D. F.; Cloft, H. J., Subarachnoid hemorrhage incidence in the United States does not vary with season or temperature. AJNR Am J Neuroradiol 2012, 33, (9), 1663-8.

35. de Rooij, N. K.; Linn, F. H.; van der Plas, J. A.; Algra, A.; Rinkel, G. J., Incidence of subarachnoid haemorrhage: a systematic review with emphasis on region, age, gender and time trends. J Neurol Neurosurg Psychiatry 2007, 78, (12), 1365-72.

36. Teunissen, L. L.; Rinkel, G. J. E.; Algra, A.; van Gijn, J., Risk Factors for Subarachnoid Hemorrhage. A Systematic Review 1996, 27, (3), 544-549.

37. van Gijn, J.; Kerr, R. S.; Rinkel, G. J., Subarachnoid haemorrhage. Lancet 2007, 369, (9558), 306-18.

38. Molyneux, A. J.; Kerr, R. S.; Yu, L. M.; Clarke, M.; Sneade, M.; Yarnold, J. A.; Sandercock, P., International subarachnoid aneurysm trial (ISAT) of neurosurgical clipping versus endovascular coiling in 2143 patients with ruptured intracranial aneurysms: a randomised comparison of effects on survival, dependency, seizures, rebleeding, subgroups, and aneurysm occlusion. Lancet 2005, 366, (9488), 809-17.

39. Inagawa, T., Trends in Incidence and Case Fatality Rates of Aneurysmal Subarachnoid Hemorrhage in Izumo City, Japan, Between 1980–1989 and 1990–1998. Stroke 2001, 32, (7), 1499-1507.

40. Cahill, J.; Zhang, J. H., Subarachnoid Hemorrhage. Is It Time for a New Direction? 2009, 40, (3 suppl 1), S86-S87.

41. Linn, F. H.; Rinkel, G. J.; Algra, A.; van Gijn, J., Headache characteristics in subarachnoid haemorrhage and benign thunderclap headache. J Neurol Neurosurg Psychiatry 1998, 65, (5), 791-3.

42. Raper, D. M. S.; Starke, R. M.; Komotar, R. J.; Allan, R.; Connolly, E. S., Jr., Seizures After Aneurysmal Subarachnoid Hemorrhage: A Systematic Review of Outcomes. World Neurosurgery 79, (5), 682-690.

43. De Marchis, G. M.; Pugin, D.; Meyers, E.; Velasquez, A.; Suwatcharangkoon, S.; Park, S.; Falo, M. C.; Agarwal, S.; Mayer, S.; Schmidt, J. M.; Connolly, E. S.; Claassen, J., Seizure burden in subarachnoid hemorrhage associated with functional and cognitive outcome. Neurology 2016, 86, (3), 253-60.

44. Backes, D.; Rinkel, G. J. E.; Sturkenboom, A. J. M.; Vergouwen, M. D. I., Time-dependent test characteristics of neck stiffness in patients suspected of nontraumatic subarachnoid haemorrhage. Journal of the Neurological Sciences 355, (1), 186-188.

45. Dubosh, N. M.; Bellolio, M. F.; Rabinstein, A. A.; Edlow, J. A., Sensitivity of Early Brain Computed Tomography to Exclude Aneurysmal Subarachnoid Hemorrhage. A Systematic Review and Meta-Analysis 2016.

46. Carpenter, C. R.; Hussain, A. M.; Ward, M. J.; Zipfel, G. J.; Fowler, S.; Pines, J. M.; Sivilotti, M. L., Spontaneous Subarachnoid Hemorrhage: A Systematic Review and Meta-analysis Describing the Diagnostic Accuracy of History, Physical Examination, Imaging, and Lumbar Puncture With an Exploration of Test Thresholds. Acad Emerg Med 2016, 23, (9), 963-1003.

47. Taylor, R. A.; Singh Gill, H.; Marcolini, E. G.; Meyers, H. P.; Faust, J. S.; Newman, D. H., Determination of a Testing Threshold for Lumbar Puncture in the Diagnosis of Subarachnoid Hemorrhage after a Negative Head Computed Tomography: A Decision Analysis. Acad Emerg Med 2016, 23, (10), 1119-1127.

48. van Gijn, J.; Rinkel, G. J. E., Subarachnoid haemorrhage: diagnosis, causes and management. Brain 2001, 124, (2), 249-278.

49. Premananda, R. M.; Ramesh, N.; Hillol, K. P., Functional outcome of microsurgical clipping compared to endovascular coiling. The Medical journal of Malaysia 2012, 67, (6), 585-590.

50. Citerio, G.; Gaini, S. M.; Tomei, G.; Stocchetti, N., Management of 350 aneurysmal subarachnoid hemorrhages in 22 Italian neurosurgical centers. Intensive Care Med 2007, 33, (9), 1580-6.

51. Koh, K. M.; Ng, Z.; Low, S. Y.; Chua, H. Z.; Chou, N.; Low, S. W.; Yeo, T. T., Management of ruptured intracranial aneurysms in the post-ISAT era: outcome of surgical clipping versus endovascular coiling in a Singapore tertiary institution. Singapore Med J 2013, 54, (6), 332-8.

52. Tholance, Y.; Barcelos, G. K.; Perret-Liaudet, A.; Omar, E.; Carrillon, R.; Grousson, S.; Lieutaud, T.; Dailler, F.; Marinesco, S., Placing intracerebral probes to optimise detection of delayed cerebral ischemia and allow for the prediction of patient outcome in aneurysmal subarachnoid haemorrhage. J Cereb Blood Flow Metab 2016.

53. Carr, K. R.; Zuckerman, S. L.; Mocco, J., Inflammation, Cerebral Vasospasm, and Evolving Theories of Delayed Cerebral Ischemia. Neurol Res Int. 2013;2013:506584. Epub 2013 Aug 22.

54. Dhar, R.; Diringer, M. N., The burden of the systemic inflammatory response predicts vasospasm and outcome after subarachnoid hemorrhage. Neurocrit Care 2008, 8, (3), 404-12.

55. Provencio, J. J., Inflammation in subarachnoid hemorrhage and delayed deterioration associated with vasospasm: a review. Acta Neurochir Suppl 2013, 115, 233-8.

56. Yoshimoto, Y.; Tanaka, Y.; Hoya, K., Acute systemic inflammatory response syndrome in subarachnoid hemorrhage. Stroke 2001, 32, (9), 1989-93.

57. Frontera, J. A.; Fernandez, A.; Schmidt, J. M.; Claassen, J.; Wartenberg, K. E.; Badjatia, N.; Connolly, E. S.; Mayer, S. A., Defining vasospasm after subarachnoid hemorrhage: what is the most clinically relevant definition? Stroke 2009, 40, (6), 1963-8.

58. Kimball, M. M.; Velat, G. J.; Hoh, B. L., Critical care guidelines on the endovascular management of cerebral vasospasm. Neurocrit Care 2011, 15, (2), 336-41.

59. Furlan, A. J., Endovascular Therapy for Stroke — It's about Time. New England Journal of Medicine 2015, 372, (24), 2347-2349.

60. Mehta, Y.; Gupta, A.; Todi, S.; Myatra, S. N.; Samaddar, D. P.; Patil, V.; Bhattacharya, P. K.; Ramasubban, S., Guidelines for prevention of hospital acquired infections. Indian Journal of Critical Care Medicine : Peer-reviewed, Official Publication of Indian Society of Critical Care Medicine 2014, 18, (3), 149-163.

61. Germanwala, A. V.; Huang, J.; Tamargo, R. J., Hydrocephalus After Aneurysmal Subarachnoid Hemorrhage. Neurosurgery Clinics 21, (2), 263-270.

62. Grasso, G.; Alafaci, C.; Macdonald, R. L., Management of aneurysmal subarachnoid hemorrhage: State of the art and future perspectives. Surg Neurol Int 2017, 8, 11.

63. Aronson, J. K., Biomarkers and surrogate endpoints. British Journal of Clinical Pharmacology 2005, 59, (5), 491-494.

64. Strimbu, K.; Tavel, J. A., What are Biomarkers? Current opinion in HIV and AIDS 2010, 5, (6), 463-466.

65. Nalejska, E.; Mączyńska, E.; Lewandowska, M. A., Prognostic and Predictive Biomarkers: Tools in Personalized Oncology. Molecular Diagnosis & Therapy 2014, 18, (3), 273-284.

66. Frank, R.; Hargreaves, R., Clinical biomarkers in drug discovery and development. Nat Rev Drug Discov 2003, 2, (7), 566-580.

67. Jickling, G. C.; Sharp, F. R., Blood biomarkers of ischemic stroke. Neurotherapeutics : the journal of the American Society for Experimental NeuroTherapeutics 2011, 8, (3), 349-360.

68. Montaner, J., Stroke biomarkers: Can they help us to guide stroke thrombolysis? Drug News Perspect 2006, 19, (9), 523-32.

69. Hirano, K.; Takashima, S.; Dougu, N.; Taguchi, Y.; Nukui, T.; Konishi, H.; Toyoda, S.; Kitajima, I.; Tanaka, K., Study of hemostatic biomarkers in acute ischemic stroke by clinical subtype. J Stroke Cerebrovasc Dis 2012, 21, (5), 404-10.

70. Kaura, V.; Bonner, S., Subarachnoid haemorrhage: Early clinical indicators and biomarkers. Trends in Anaesthesia and Critical Care 2012, 2, (1), 42-47.

71. Elkind, M. S. V., Inflammatory markers and stroke. Current Cardiology Reports 2008, 11, (1), 12.

72. Elkind, M. S.; Luna, J. M.; Coffey, C. S.; McClure, L. A.; Liu, K. M.; Spitalnik, S.; Paik, M. C.; Roldan, A.; White, C.; Hart, R.; Benavente, O., The Levels of Inflammatory Markers in the Treatment of Stroke study (LIMITS): inflammatory biomarkers as risk predictors after lacunar stroke. Int J Stroke 2010, 5, (2), 117-25.

73. del Zoppo, G. J.; Becker, K. J.; Hallenbeck, J. M., Inflammation after stroke: is it harmful? Arch Neurol 2001, 58, (4), 669-72.

74. Carr, K. R.; Zuckerman, S. L.; Mocco, J., Inflammation, cerebral vasospasm, and evolving theories of delayed cerebral ischemia. Neurol Res Int 2013, 2013, 506584.

75. Antonino, T.; Domenico Di, R.; Riccardo di, S.; Antonio, P.; Giuseppe, L., Inflammatory Cytokines in Acute Ischemic Stroke. Current Pharmaceutical Design 2008, 14, (33), 3574-3589.

76. Tyrrell, H. C. A. E. a. P. J., Inflammation and infection in clinical stroke. Journal of Cerebral Blood Flow & Metabolism 2002, 22, 1399–1419

77. Dziedzic, T.; Slowik, A.; Szczudlik, A., Interleukin-6 and Stroke: Cerebral Ischemia Versus Nonspecific Factors Influencing Interleukin-6. Stroke 2003, 34, (12), e229-e230.

78. Katsnelson, M.; Rundek, T., Chemokines and stroke: the subcellular harbingers of apoplexy? Neurology 2011, 77, (12), 1116-7.

79. Rosell, A.; Cuadrado, E.; Ortega-Aznar, A.; Hernández-Guillamon, M.; Lo, E. H.; Montaner, J., MMP-9–Positive Neutrophil Infiltration Is Associated to Blood–Brain Barrier Breakdown and Basal Lamina Type IV Collagen Degradation During Hemorrhagic Transformation After Human Ischemic Stroke. Stroke 2008, 39, (4), 1121-1126.

80. Vukasovic, I.; Tesija-Kuna, A.; Topic, E.; Supanc, V.; Demarin, V.; Petrovcic, M., Matrix metalloproteinases and their inhibitors in different acute stroke subtypes. Clin Chem Lab Med 2006, 44, (4), 428-34.

81. Simundic, A. M.; Basic, V.; Topic, E.; Demarin, V.; Vrkic, N.; Kunovic, B.; Stefanovic, M.; Begonja, A., Soluble adhesion molecules in acute ischemic stroke. Clin Invest Med 2004, 27, (2), 86-92.

82. Lad, S. P.; Hegen, H.; Gupta, G.; Deisenhammer, F.; Steinberg, G. K., Proteomic biomarker discovery in cerebrospinal fluid for cerebral vasospasm following subarachnoid hemorrhage. J Stroke Cerebrovasc Dis 2012, 21, (1), 30-41.

83. Parkinson, D.; Stephensen, S., Leukocytosis and subarachnoid hemorrhage. Surg Neurol 1984, 21, (2), 132-4.

84. Benakis, C.; Garcia-Bonilla, L.; Iadecola, C.; Anrather, J., The role of microglia and myeloid immune cells in acute cerebral ischemia. Front Cell Neurosci 2014, 8.

85. Romero, F. R.; Bertolini Ede, F.; Figueiredo, E. G.; Teixeira, M. J., Serum C-reactive protein levels predict neurological outcome after aneurysmal subarachnoid hemorrhage. Arq Neuropsiquiatr 2012, 70, (3), 202-5.

86. Brea, D.; Sobrino, T.; Blanco, M.; Fraga, M.; Agulla, J.; Rodriguez-Yanez, M.; Rodriguez-Gonzalez, R.; Perez de la Ossa, N.; Leira, R.; Forteza, J.; Davalos, A.; Castillo, J., Usefulness of haptoglobin and serum amyloid A proteins as biomarkers for atherothrombotic ischemic stroke diagnosis confirmation. Atherosclerosis 2009, 205, (2), 561-7.

87. Emsley, H. C.; Smith, C. J.; Gavin, C. M.; Georgiou, R. F.; Vail, A.; Barberan, E. M., An early and sustained peripheral inflammatory response in acute ischaemic stroke: relationships with infection and atherosclerosis. J Neuroimmunol. 2003, 139.

88. Offner, H.; Subramanian, S.; Parker, S. M.; Afentoulis, M. E.; Vandenbark, A. A.; Hurn, P. D., Experimental stroke induces massive, rapid activation of the peripheral immune system. J Cereb Blood Flow Metab 2006, 26, (5), 654-65.

89. Emsley, H. C.; Hopkins, S. J., Post-stroke immunodepression and infection: an emerging concept. Infect Disord Drug Targets 2010, 10, (2), 91-7.

90. Chamorro, A.; Urra, X.; Planas, A. M., Infection after acute ischemic stroke: a manifestation of brain-induced immunodepression. Stroke. 2007, 38.

91. Harms, H.; Grittner, U.; Droge, H.; Meisel, A., Predicting post-stroke pneumonia: the PANTHERIS score. Acta Neurol Scand 2013, 128, (3), 178-84.

92. Kalra, L.; Irshad, S.; Hodsoll, J.; Simpson, M.; Gulliford, M.; Smithard, D.; Patel, A.; Rebollo-Mesa, I., Prophylactic antibiotics after acute stroke for reducing pneumonia in patients with dysphagia (STROKE-INF): a prospective, cluster-randomised, open-label, masked endpoint, controlled clinical trial. The Lancet 2015, 386, (10006), 1835-1844.

93. Westendorp, W. F.; Vermeij, J. D.; Zock, E.; Hooijenga, I. J.; Kruyt, N. D.; Bosboom, H. J.; Kwa, V. I.; Weisfelt, M.; Remmers, M. J.; ten Houten, R.; Schreuder, A. H.; Vermeer, S. E.; van Dijk, E. J.; Dippel, D. W.; Dijkgraaf, M. G.; Spanjaard, L.; Vermeulen, M.; van der Poll, T.; Prins, J. M.; Vermeij, F. H.; Roos, Y. B.; Kleyweg, R. P.; Kerkhoff, H.; Brouwer, M. C.; Zwinderman, A. H.; van de Beek, D.; Nederkoorn, P. J., The Preventive Antibiotics in Stroke Study (PASS): a pragmatic randomised open-label masked endpoint clinical trial. Lancet 2015, 385, (9977), 1519-26.

94. Burillo, A.; Bouza, E., Use of rapid diagnostic techniques in ICU patients with infections. BMC Infectious Diseases 2014, 14, (1), 593.

95. Albrich, W. C.; Harbarth, S., Pros and cons of using biomarkers versus clinical decisions in start and stop decisions for antibiotics in the critical care setting. Intensive Care Med 2015, 41, (10), 1739-51.

96. Gumbinger, C.; Hug, A.; Murle, B.; Berger, B.; Zorn, M.; Becker, K. P.; Zimmermann, S.; Dalpke, A. H.; Veltkamp, R., Early blood-based microbiological testing is ineffective in severe stroke patients. J Neurol Sci 2013, 325, (1-2), 46-50.

97. Fluri, F.; Morgenthaler, N. G.; Mueller, B.; Christ-Crain, M.; Katan, M., Copeptin, Procalcitonin and Routine Inflammatory Markers–Predictors of Infection after Stroke. PloS one 2012, 7, (10), e48309.

98. Wartenberg, K. E.; Stoll, A.; Funk, A.; Meyer, A.; Schmidt, J. M.; Berrouschot, J., Infection after Acute Ischemic Stroke: Risk Factors, Biomarkers, and Outcome. Stroke Res Treat 2011, 2011.

99. Worthmann, H.; Tryc, A. B.; Dirks, M.; Schuppner, R.; Brand, K.; Klawonn, F.; Lichtinghagen, R.; Weissenborn, K., Lipopolysaccharide binding protein, interleukin-10, interleukin-6 and C-reactive protein blood levels in acute ischemic stroke patients with post-stroke infection. Journal of Neuroinflammation 2015, 12, (1), 13.

100. Kwon, H.-M.; Jeong, S.-W.; Lee, S.-H.; Yoon, B.-W., The pneumonia score: A simple grading scale for prediction of pneumonia after acute stroke. American Journal of Infection Control 34, (2), 64-68.

101. Chumbler, N. R.; Williams, L. S.; Wells, C. K.; Lo, A. C.; Nadeau, S.; Peixoto, A. J.; Gorman, M.; Boice, J. L.; Concato, J.; Bravata, D. M., Derivation and Validation of a Clinical System for Predicting Pneumonia in Acute Stroke. Neuroepidemiology 2010, 34, (4), 193-199.

102. Smith, C. J.; Bray, B. D.; Hoffman, A.; Meisel, A.; Heuschmann, P. U.; Wolfe, C. D.; Tyrrell, P. J.; Rudd, A. G., Can a novel clinical risk score improve pneumonia prediction in acute stroke care? A UK multicenter cohort study. J Am Heart Assoc 2015, 4, (1), e001307.

103. Hoffmann, S.; Malzahn, U.; Harms, H.; Koennecke, H. C.; Berger, K.; Kalic, M.; Walter, G.; Meisel, A.; Heuschmann, P. U., Development of a clinical score (A2DS2) to predict pneumonia in acute ischemic stroke. Stroke 2012, 43, (10), 2617-23.

104. Li, Y.; Song, B.; Fang, H.; Gao, Y.; Zhao, L.; Xu, Y., External validation of the A2DS2 score to predict stroke-associated pneumonia in a Chinese population: a prospective cohort study. PLoS One 2014, 9, (10), e109665.

105. Craft, G. E.; Chen, A.; Nairn, A. C., Recent advances in quantitative neuroproteomics. Methods 2013, 61, (3), 186-218.

106. Rabilloud, T.; Lelong, C., Two-dimensional gel electrophoresis in proteomics: A tutorial. Journal of Proteomics 2011, 74, (10), 1829-1841.

107. Bunai, K.; Yamane, K., Effectiveness and limitation of two-dimensional gel electrophoresis in bacterial membrane protein proteomics and perspectives. Journal of Chromatography B 2005, 815, (1–2), 227-236.

108. Fenn, J.; Mann, M.; Meng, C.; Wong, S.; Whitehouse, C., Electrospray ionization for mass spectrometry of large biomolecules. Science 1989, 246, (4926), 64-71.

109. Karas, M.; Hillenkamp, F., Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem 1988, 60, (20), 2299-301.

110. Bantscheff, M.; Schirle, M.; Sweetman, G.; Rick, J.; Kuster, B., Quantitative mass spectrometry in proteomics: a critical review. Analytical and Bioanalytical Chemistry 2007, 389, (4), 1017-1031.

111. Asara, J.; Christofk, H.; Freimark, L.; Cantley, L., A label-free quantification method by MS/MS TIC compared to SILAC and spectral counting in a proteomics screen. Proteomics 2008, 8, (5), 994-999.

112. Morton, T. H., Isotopic Labelling in Mass Spectrometry* A2 - Lindon, John C. In Encyclopedia of Spectroscopy and Spectrometry (Second Edition), Academic Press: Oxford, 2010; pp 1237-1246.

113. Xiao, Z.; Veenstra, T. D., Comparison of protein expression by isotope-coded affinity tag labeling. Methods Mol Biol 2008, 428, 181-92.

114. Venable, J. D.; Dong, M.-Q.; Wohlschlegel, J.; Dillin, A.; Yates, J. R., Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra. Nat Meth 2004, 1, (1), 39-45.

115. Ross, P. L.; Huang, Y. N.; Marchese, J. N.; Williamson, B.; Parker, K.; Hattan, S.; Khainovski, N.; Pillai, S.; Dey, S.; Daniels, S.; Purkayastha, S.; Juhasz, P.; Martin, S.; Bartlet-Jones, M.; He, F.; Jacobson, A.; Pappin, D. J., Multiplexed Protein Quantitation in Saccharomyces cerevisiae Using Amine-reactive Isobaric Tagging Reagents. Molecular & Cellular Proteomics 2004, 3, (12), 1154-1169.

116. Evans, C.; Noirel, J.; Ow, S. Y.; Salim, M.; Pereira-Medrano, A. G.; Couto, N.; Pandhal, J.; Smith, D.; Pham, T. K.; Karunakaran, E.; Zou, X.; Biggs, C. A.; Wright, P. C., An insight into iTRAQ: where do we stand now? Anal Bioanal Chem 2012, 404, (4), 1011-27.

117. Kani, K., Quantitative Proteomics Using SILAC. Methods Mol Biol 2017, 1550, 171-184.

118. Mitchell, P., Proteomics retrenches. Nat Biotech 2010, 28, (7), 665-670.

119. Hortin, G. L.; Sviridov, D., The dynamic range problem in the analysis of the plasma proteome. Journal of Proteomics 2010, 73, (3), 629-636.

120. Hunter, D. J.; Losina, E.; Guermazi, A.; Burstein, D.; Lassere, M. N.; Kraus, V., A pathway and approach to biomarker validation and qualification for osteoarthritis clinical trials. Curr Drug Targets 2010, 11, (5), 536-45.

121. Whiteaker, J. R.; Lin, C.; Kennedy, J.; Hou, L.; Trute, M.; Sokal, I.; Yan, P.; Schoenherr, R. M.; Zhao, L.; Voytovich, U. J.; Kelly-Spratt, K. S.; Krasnoselsky, A.; Gafken, P. R.; Hogan, J. M.; Jones, L. A.; Wang, P.; Amon, L.; Chodosh, L. A.; Nelson, P. S.; McIntosh, M. W.; Kemp, C. J.; Paulovich, A. G., A targeted proteomics-based pipeline for verification of biomarkers in plasma. Nat Biotech 2011, 29, (7), 625-634.

122. Cummings, J.; Raynaud, F.; Jones, L.; Sugar, R.; Dive, C., Fit-for-purpose biomarker method validation for application in clinical trials of anticancer drugs. Br J Cancer 2010, 103, (9), 1313-1317.

123. Haverland, N.; Pottiez, G.; Wiederin, J.; Ciborowski, P., Immunoreactivity of anti-gelsolin antibodies: implications for biomarker validation. Journal of Translational Medicine 2010, 8, (1), 137.

124. Bourmaud, A.; Gallien, S.; Domon, B., Parallel reaction monitoring using quadrupole-Orbitrap mass spectrometer: Principle and applications. Proteomics 2016, 16, (15-16), 2146-59.

125. Rauniyar, N., Parallel Reaction Monitoring: A Targeted Experiment Performed Using High Resolution and High Mass Accuracy Mass Spectrometry. Int J Mol Sci 2015, 16, (12), 28566-81.

126. Cummings, J.; Ward, T. H.; Greystoke, A.; Ranson, M.; Dive, C., Biomarker method validation in anticancer drug development. Br J Pharmacol 2008, 153, (4), 646-56.

127. Robin, X.; Turck, N.; Hainard, A.; Tiberti, N.; Lisacek, F.; Sanchez, J. C.; Muller, M., pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011, 12, (77), 1471-2105.

128. Hajian-Tilaki, K., Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation. Caspian J Intern Med 2013, 4, (2), 627-35.

129. Robin, X.; Turck, N.; Hainard, A.; Tiberti, N.; Lisacek, F.; Sanchez, J.-C.; Müller, M., PanelomiX: A threshold-based algorithm to create panels of biomarkers. Translational Proteomics 2013, 1, (1), 57-64.

130. Varghese, S.; Lao–Sirieix, P.; Fitzgerald, R. C., Identification and Clinical Implementation of Biomarkers for Barrett's Esophagus. Gastroenterology 2012, 142, (3), 435-441.e2.

131. Mischak, H.; Ioannidis, J. P.; Argiles, A.; Attwood, T. K.; Bongcam-Rudloff, E.; Broenstrup, M.; Charonis, A.; Chrousos, G. P.; Delles, C.; Dominiczak, A.; Dylag, T.; Ehrich, J.; Egido, J.; Findeisen, P.; Jankowski, J.; Johnson, R. W.; Julien, B. A.; Lankisch, T.; Leung, H. Y.; Maahs, D.; Magni, F.; Manns, M. P.; Manolis, E.; Mayer, G.; Navis, G.; Novak, J.; Ortiz, A.; Persson, F.; Peter,

K.; Riese, H. H.; Rossing, P.; Sattar, N.; Spasovski, G.; Thongboonkerd, V.; Vanholder, R.; Schanstra, J. P.; Vlahou, A., Implementation of proteomic biomarkers: making it work. Eur J Clin Invest 2012, 42, (9), 1027-36.

132. Shevchenko, G.; Konzer, A.; Musunuri, S.; Bergquist, J., Neuroproteomics tools in clinical practice. Biochim Biophys Acta 2015, 1854, (7), 705-17.

133. Wang, K. K. W.; Montaner, J., Neuroproteomics 101. Translational Proteomics 2014, 3, A1-A2.

134. Liu, T.; Hu, J.; Li, H., iTRAQ-based Shotgun Neuroproteomics. Methods in molecular biology (Clifton, N.J.) 2009, 566, 201-216.

135. Rauniyar, N.; Yates, J. R., 3rd, Isobaric labeling-based relative quantification in shotgun proteomics. J Proteome Res 2014, 13, (12), 5293-309.

136. Dayon, L.; Turck, N.; Scherl, A.; Hochstrasser, D. F.; Burkhard, P. R.; Sanchez, J. C., From relative to absolute quantification of tryptic peptides with tandem mass tags: application to cerebrospinal fluid. Chimia (Aarau) 2010, 64, (3), 132-5.

137. Thompson, A.; Schafer, J.; Kuhn, K.; Kienle, S.; Schwarz, J.; Schmidt, G.; Neumann, T.; Johnstone, R.; Mohammed, A. K.; Hamon, C., Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 2003, 75, (8), 1895-904.

138. Chiva, C.; Sabido, E., HCD-only fragmentation method balances peptide identification and quantitation of TMT-labeled samples in hybrid linear ion trap/orbitrap mass spectrometers. J Proteomics 2014, 96, 263-70.

139. Dayon, L.; Pasquarello, C.; Hoogland, C.; Sanchez, J. C.; Scherl, A., Combining low- and high-energy tandem mass spectra for optimized peptide quantification with isobaric tags. J Proteomics 2010, 73, (4), 769-77.

140. Ye, H.; Boyne, M. T., 2nd; Buhse, L. F.; Hill, J., Direct approach for qualitative and quantitative characterization of glycoproteins using tandem mass tags and an LTQ Orbitrap XL electron transfer dissociation hybrid mass spectrometer. Anal Chem 2013, 85, (3), 1531-9.

141. Viner, R. I.; Zhang, T.; Second, T.; Zabrouskov, V., Quantification of post-translationally modified peptides of bovine alpha-crystallin using tandem mass tags and electron transfer dissociation. J Proteomics 2009, 72, (5), 874-85.

142. Dayon, L.; Hainard, A.; Licker, V.; Turck, N.; Kuhn, K.; Hochstrasser, D. F.; Burkhard, P. R.; Sanchez, J. C., Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. Anal Chem 2008, 80, (8), 2921-31.

143. Ballard, C.; Gauthier, S.; Corbett, A.; Brayne, C.; Aarsland, D.; Jones, E., Alzheimer's disease. Lancet 2011, 377, (9770), 1019-31.

144. Lv, J.; Ma, S.; Zhang, X.; Zheng, L.; Ma, Y.; Zhao, X.; Lai, W.; Shen, H.; Wang, Q.; Ji, J., Quantitative proteomics reveals that PEA15 regulates astroglial Aβ phagocytosis in an Alzheimer's disease mouse model. Journal of Proteomics 2014, 110, 45-58.

145. Liu, Y.; Qing, H.; Deng, Y., Biomarkers in Alzheimer's disease analysis by mass spectrometry-based proteomics. Int J Mol Sci 2014, 15, (5), 7865-82.

146. Licker, V.; Turck, N.; Kovari, E.; Burkhardt, K.; Cote, M.; Surini-Demiri, M.; Lobrinus, J. A.; Sanchez, J. C.; Burkhard, P. R., Proteomic analysis of human substantia nigra identifies novel candidates involved in Parkinson's disease pathogenesis. Proteomics 2014, 14, (6), 784-94.

147. Dayon, L.; Turck, N.; García-Berrocoso, T.; Walter, N.; Burkhard, P. R.; Vilalta, A.; Sahuquillo, J.; Montaner, J.; Sanchez, J.-C., Brain Extracellular Fluid Protein Changes in Acute Stroke Patients. Journal of Proteome Research 2011, 10, (3), 1043-1051.

148. Laban, K. G.; Rinkel, G. J. E.; Vergouwen, M. D. I., Nosocomial infections after aneurysmal subarachnoid hemorrhage: time course and causative pathogens. International Journal of Stroke 2015, 10, (5), 763-766.

149. Azurmendi, L.; Degos, V.; Tiberti, N.; Kapandji, N.; Sanchez, P.; Sarrafzadeh, A.; Puybasset, L.; Turck, N.; Sanchez, J.-C., Measuring Serum Amyloid A for Infection Prediction in Aneurysmal Subarachnoid Hemorrhage. Journal of Proteome Research 2015, 14, (9), 3948-3956.

150. Nunez Galindo, A.; Kussmann, M.; Dayon, L., Proteomics of Cerebrospinal Fluid: Throughput and Robustness Using a Scalable Automated Analysis Pipeline for Biomarker Discovery. Anal Chem 2015, 87, (21), 10755-61.

151. Yu, L. R.; Conrads, T. P.; Uo, T.; Kinoshita, Y.; Morrison, R. S.; Lucas, D. A.; Chan, K. C.; Blonder, J.; Issaq, H. J.; Veenstra, T. D., Global analysis of the cortical neuron proteome. Mol Cell Proteomics 2004, 3, (9), 896-907.

152. Giron, P.; Dayon, L.; Turck, N.; Hoogland, C.; Sanchez, J. C., Quantitative analysis of human cerebrospinal fluid proteins using a combination of cysteine tagging and amine-reactive isobaric labeling. J Proteome Res 2011, 10, (1), 249-58.

153. Friedman, D. B., Quantitative proteomics for two-dimensional gels using difference gel electrophoresis. Methods Mol Biol 2007, 367, 219-39.

154. Deutsch, E. W.; Overall, C. M.; Van Eyk, J. E.; Baker, M. S.; Paik, Y. K.; Weintraub, S. T.; Lane, L.; Martens, L.; Vandenbrouck, Y.; Kusebauch, U.; Hancock, W. S.; Hermjakob, H.; Aebersold, R.; Moritz, R. L.; Omenn, G. S., Human Proteome Project Mass Spectrometry Data Interpretation Guidelines 2.1. J Proteome Res 2016, 15, (11), 3961-3970.

155. Frontera, J. A.; Fernandez, A.; Schmidt, J. M.; Claassen, J.; Wartenberg, K. E.; Badjatia, N.; Parra, A.; Connolly, E. S.; Mayer, S. A., Impact of nosocomial infectious complications after subarachnoid hemorrhage. Neurosurgery 2008, 62, (1), 80-7.

156. Hilker, R.; Poetter, C.; Findeisen, N.; Sobesky, J.; Jacobs, A.; Neveling, M.; Heiss, W.-D., Nosocomial Pneumonia After Acute Stroke: Implications for Neurological Intensive Care Medicine. Stroke 2003, 34, (4), 975-981.

157. Douds, G. L.; Tadzong, B.; Agarwal, A. D.; Krishnamurthy, S.; Lehman, E. B.; Cockroft, K. M., Influence of Fever and Hospital-Acquired Infection on the Incidence of Delayed Neurological Deficit and Poor Outcome after Aneurysmal Subarachnoid Hemorrhage. Neurology Research International 2012, 2012, 6.

158. Ulm, L.; Ohlraun, S.; Harms, H.; Hoffmann, S.; Klehmet, J.; Ebmeyer, S.; Hartmann, O.; Meisel, C.; Anker, S. D.; Meisel, A., STRoke Adverse outcome is associated WIth NoSocomial Infections (STRAWINSKI): procalcitonin ultrasensitive-guided antibacterial therapy in severe ischaemic stroke patients - rationale and protocol for a randomized controlled trial. Int J Stroke 2013, 8, (7), 598-603.

159. Harms, H.; Prass, K.; Meisel, C.; Klehmet, J.; Rogge, W.; Drenckhahn, C.; Göhler, J.; Bereswill, S.; Göbel, U.; Wernecke, K. D.; Wolf, T.; Arnold, G.; Halle, E.; Volk, H.-D.; Dirnagl, U.; Meisel, A., Preventive Antibacterial Therapy in Acute Ischemic Stroke: A Randomized Controlled Trial. PloS one 2008, 3, (5), e2158.

160. Biomarkers: Potential Uses and Limitations.

161. Hong, C. M.; Tosun, C.; Kurland, D. B.; Gerzanich, V.; Schreibman, D.; Simard, J. M., Biomarkers as outcome predictors in subarachnoid hemorrhage--a systematic review. Biomarkers 2014, 19, (2), 95-108.

162. Rallidis, L. S.; Vikelis, M.; Panagiotakos, D. B.; Rizos, I.; Zolindaki, M. G.; Kaliva, K.; Kremastinos, D. T., Inflammatory markers and in-hospital mortality in acute ischaemic stroke. Atherosclerosis 2006, 189, (1), 193-197.

163. Leire Azurmendi, V. D., Natalia Tiberti, Natacha Kapandji, Paola Sanchez, Asita Sarrafzadeh, Louis Puybasset, Natacha Turck, Jean-Charles Sanchez, Neopterin plasma levels correlate with infection and long-term outcome in aneurysmal subarachnoid haemorrhage. Journal of Neurosurgery - [Epub ahead of print] 2015.

164. Azurmendi, L.; Degos, V.; Tiberti, N.; Kapandji, N.; Sanchez-Pena, P.; Sarrafzadeh, A.; Puybasset, L.; Turck, N.; Sanchez, J. C., Neopterin plasma concentrations in patients with aneurysmal subarachnoid hemorrhage: correlation with infection and long-term outcome. J Neurosurg 2015, 1-13.

165. Turck, N.; Vutskits, L.; Sanchez-Pena, P.; Robin, X.; Hainard, A.; Gex-Fabry, M.; Fouda, C.; Bassem, H.; Mueller, M.; Lisacek, F.; Puybasset, L.; Sanchez, J.-C., A multiparameter panel method for outcome prediction following aneurysmal subarachnoid hemorrhage. Intensive Care Medicine 2010, 36, (1), 107-115.

166. Teasdale, G. M.; Drake, C. G.; Hunt, W.; Kassell, N.; Sano, K.; Pertuiset, B.; De Villiers, J. C., A universal subarachnoid hemorrhage scale: report of a committee of the World Federation of Neurosurgical Societies. Journal of Neurology, Neurosurgery, and Psychiatry 1988, 51, (11), 1457.

167. Teasdale, G.; Murray, G.; Parker, L.; Jennett, B., Adding up the Glasgow Coma Score. Acta Neurochir Suppl 1979, 28, (1), 13-6.

168. Claassen, J.; Bernardini, G. L.; Kreiter, K.; Bates, J.; Du, Y. E.; Copeland, D.; Connolly, E. S.; Mayer, S. A., Effect of Cisternal and Ventricular Blood on Risk of Delayed Cerebral Ischemia After Subarachnoid Hemorrhage:: The Fisher Scale Revisited. Stroke 2001, 32, (9), 2012-2020.

169. Jennett, B.; Bond, M., Assessment of outcome after severe brain damage. Lancet 1975, 1, (7905), 480-4.

170. Calandra, T.; Cohen, J., The international sepsis forum consensus conference on definitions of infection in the intensive care unit. Crit Care Med 2005, 33, (7), 1538-48.

171. van Bussel, B. C. T.; Henry, R. M. A.; Schalkwijk, C. G.; Ferreira, I.; Feskens, E. J. M.; Streppel, M. T.; Smulders, Y. M.; Twisk, J. W. R.; Stehouwer, C. D. A., Fish Consumption in Healthy Adults Is Associated with Decreased Circulating Biomarkers of Endothelial Dysfunction and Inflammation during a 6-Year Follow-Up. The Journal of Nutrition 2011, 141, (9), 1719-1725.

172. Klehmet, J.; Harms, H.; Richter, M.; Prass, K.; Volk, H. D.; Dirnagl, U.; Meisel, A.; Meisel, C., Stroke-induced immunodepression and post-stroke infections: lessons from the preventive antibacterial therapy in stroke trial. Neuroscience 2009, 158, (3), 1184-93.

173. Yang, Y. H.; Dudoit, S.; Luu, P.; Lin, D. M.; Peng, V.; Ngai, J.; Speed, T. P., Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Research 2002, 30, (4), e15.

174. Grabska, K.; Gromadzka, G.; #380; yna; Cz; #322; onkowska, A., Infections and Ischemic Stroke Outcome. Neurology Research International 2011, 2011.

175. Savardekar, A.; Gyurmey, T.; Agarwal, R.; Podder, S.; Mohindra, S.; Gupta, S. K.; Chhabra, R., Incidence, risk factors, and outcome of postoperative pneumonia after microsurgical clipping of ruptured intracranial aneurysms. Surg Neurol Int 2013, 4, 24.

176. Cinotti, R.; Dordonnat-Moynard, A.; Feuillet, F.; Roquilly, A.; Rondeau, N.; Lepelletier, D.; Caillon, J.; Asseray, N.; Blanloeil, Y.; Rozec, B.; Asehnoune, K., Risk factors and pathogens

involved in early ventilator-acquired pneumonia in patients with severe subarachnoid hemorrhage. Eur J Clin Microbiol Infect Dis 2014, 33, (5), 823-30.

177. Sarrafzadeh, A.; Schlenk, F.; Meisel, A.; Dreier, J.; Vajkoczy, P.; Meisel, C., Immunodepression after aneurysmal subarachnoid hemorrhage. Stroke 2011, 42, (1), 53-8.

178. Maiuri, F.; Gallicchio, B.; Donati, P.; Carandente, M., The blood leukocyte count and its prognostic significance in subarachnoid hemorrhage. J Neurosurg Sci 1987, 31, (2), 45-8.

179. Urieli-Shoval, S.; Linke, R. P.; Matzner, Y., Expression and function of serum amyloid A, a major acute-phase protein, in normal and disease states. Curr Opin Hematol 2000, 7, (1), 64-9.

180. Casl, M. T.; Rogina, B.; Glojnaric-Spasic, I.; Minigo, H.; Planinc-Peraica, A.; Jaksic, B., The differential diagnostic capacity of serum amyloid A protein between infectious and non-infectious febrile episodes of neutropenic patients with acute leukemia. Leuk Res 1994, 18, (9), 665-70.

181. Miwata, H.; Yamada, T.; Okada, M.; Kudo, T.; Kimura, H.; Morishima, T., Serum amyloid A protein in acute viral infections. Archives of Disease in Childhood 1993, 68, (2), 210-214.

182. Nakayama, T.; Sonoda, S.; Urano, T.; Yamada, T.; Okada, M., Monitoring both serum amyloid protein A and C-reactive protein as inflammatory markers in infectious diseases. Clinical Chemistry 1993, 39, (2), 293-7.

183. Lannergard, A.; Larsson, A.; Kragsbjerg, P.; Friman, G., Correlations between serum amyloid A protein and C-reactive protein in infectious diseases. Scand J Clin Lab Invest 2003, 63, (4), 267-72.

184. Du Clos, T. W., Function of C-reactive protein. Ann Med 2000, 32, (4), 274-8.

185. Berdowska, A.; Zwirska-Korczala, K., Neopterin measurement in clinical diagnosis. J Clin Pharm Ther 2001, 26, (5), 319-29.

186. Murr, C.; Widner, B.; Wirleitner, B.; Fuchs, D., Neopterin as a marker for immune system activation. Curr Drug Metab 2002, 3, (2), 175-87.

187. Mathiesen, T.; Fuchs, D.; Wachter, H.; von Holst, H., Increased CSF neopterin levels in subarachnoid hemorrhage. J Neurosurg 1990, 73, (1), 69-71.

188. Global, regional, and national age–sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. The Lancet 385, (9963), 117-171.

189. Truelsen, T.; Piechowski-Jozwiak, B.; Bonita, R.; Mathers, C.; Bogousslavsky, J.; Boysen, G., Stroke incidence and prevalence in Europe: a review of available data. Eur J Neurol 2006, 13, (6), 581-98.

190. Warlow, C.; Sudlow, C.; Dennis, M.; Wardlaw, J.; Sandercock, P., Stroke. Lancet 2003, 362, (9391), 1211-24.

191. Langhorne, P.; Stott, D. J.; Robertson, L.; MacDonald, J.; Jones, L.; McAlpine, C.; Dick, F.; Taylor, G. S.; Murray, G., Medical Complications After Stroke. A Multicenter Study 2000, 31, (6), 1223-1229.

192. Tirschwell, D. L.; Kukull, W. A.; Longstreth Jr, W. T., Medical complications of ischemic stroke and length of hospital stay: Experience in Seattle, Washington. Journal of Stroke and Cerebrovascular Diseases 1999, 8, (5), 336-343.

193. Al-Khaled, M.; Matthis, C.; Eggers, J., Predictors of In-hospital Mortality and the Risk of Symptomatic Intracerebral Hemorrhage after Thrombolytic Therapy with Recombinant Tissue Plasminogen Activator in Acute Ischemic Stroke. Journal of Stroke and Cerebrovascular Diseases 23, (1), 7-11.

194. Hug, A.; Murle, B.; Dalpke, A.; Zorn, M.; Liesz, A.; Veltkamp, R., Usefulness of serum procalcitonin levels for the early diagnosis of stroke-associated respiratory tract infections. Neurocrit Care 2011, 14, (3), 416-22.

195. Chamorro, Á.; Urra, X.; Planas, A. M., Infection After Acute Ischemic Stroke. A Manifestation of Brain-Induced Immunodepression 2007, 38, (3), 1097-1103.

196. Weimar, C.; Roth, M. P.; Zillessen, G.; Glahn, J.; Wimmer, M. L.; Busse, O.; Haberl, R. L.; Diener, H. C., Complications following acute ischemic stroke. Eur Neurol 2002, 48, (3), 133-40.

197. Westendorp, W. F.; Nederkoorn, P. J.; Vermeij, J. D.; Dijkgraaf, M. G.; van de Beek, D., Post-stroke infection: a systematic review and meta-analysis. BMC Neurol 2011, 11, 110.

198. Marrie, T. J.; Wu, L., Factors influencing in-hospital mortality in community-acquired pneumonia: a prospective study of patients not initially admitted to the ICU. Chest 2005, 127, (4), 1260-70.

199. Dirnagl, U.; Klehmet, J.; Braun, J. S.; Harms, H.; Meisel, C.; Ziemssen, T.; Prass, K.; Meisel, A., Stroke-Induced Immunodepression: Experimental Evidence and Clinical Relevance. Stroke 2007, 38, (2), 770-773.

200. Ji, R.; Shen, H.; Pan, Y.; Wang, P.; Liu, G.; Wang, Y.; Li, H.; Wang, Y., Novel risk score to predict pneumonia after acute ischemic stroke. Stroke 2013, 44, (5), 1303-9.

201. Finlayson, O.; Kapral, M.; Hall, R.; Asllani, E.; Selchen, D.; Saposnik, G., Risk factors, inpatient care, and outcomes of pneumonia after ischemic stroke. Neurology 2011, 77, (14), 1338-45.

202. Garner, J. S.; Jarvis, W. R.; Emori, T. G.; Horan, T. C.; Hughes, J. M., CDC definitions for nosocomial infections, 1988. American Journal of Infection Control 16, (3), 128-140.

203. Dayon, L.; Turck, N.; Kienle, S.; Schulz-Knappe, P.; Hochstrasser, D. F.; Scherl, A.; Sanchez, J. C., Isobaric tagging-based selection and quantitation of cerebrospinal fluid tryptic peptides with reporter calibration curves. Anal Chem 2010, 82, (3), 848-58.

204. Dayon, L.; Turck, N.; Scherl, A.; Hochstrasser, D. F.; Burkhard, P. R.; Sanchez, J.-C., From Relative to Absolute Quantification of Tryptic Peptides with Tandem Mass Tags: Application to Cerebrospinal Fluid. CHIMIA International Journal for Chemistry 2010, 64, (3), 132-135.

205. Dayon, L.; Pasquarello, C.; Hoogland, C.; Sanchez, J.-C.; Scherl, A., Combining low- and high-energy tandem mass spectra for optimized peptide quantification with isobaric tags. Journal of Proteomics 2010, 73, (4), 769-777.

206. Gluck, F.; Hoogland, C.; Antinori, P.; Robin, X.; Nikitin, F.; Zufferey, A.; Pasquarello, C.; Fétaud, V.; Dayon, L.; Müller, M.; Lisacek, F.; Geiser, L.; Hochstrasser, D.; Sanchez, J.-C.; Scherl, A., EasyProt — An easy-to-use graphical platform for proteomics data analysis. Journal of Proteomics 2013, 79, (0), 146-160.

207. Elias, J. E.; Gygi, S. P., Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods 2007, 4, (3), 207-14.

208. Breitwieser, F. P.; Muller, A.; Dayon, L.; Kocher, T.; Hainard, A.; Pichler, P.; Schmidt-Erfurth, U.; Superti-Furga, G.; Sanchez, J. C.; Mechtler, K.; Bennett, K. L.; Colinge, J., General statistical modeling of data from protein relative expression isobaric tags. J Proteome Res 2011, 10, (6), 2758-66.

209. Tiberti, N.; Hainard, A.; Lejon, V.; Robin, X.; Ngoyi, D. M.; Turck, N.; Matovu, E.; Enyaru, J.; Ndung'u, J. M.; Scherl, A.; Dayon, L.; Sanchez, J.-C., Discovery and Verification of Osteopontin and Beta-2-microglobulin as Promising Markers for Staging Human African Trypanosomiasis. Molecular & Cellular Proteomics : MCP 2010, 9, (12), 2783-2795.

210. Tan, H. T.; Tan, S.; Lin, Q.; Lim, T. K.; Hew, C. L.; Chung, M. C., Quantitative and temporal proteome analysis of butyrate-treated colorectal cancer cells. Mol Cell Proteomics 2008, 7, (6), 1174-85.

211. Salvisberg, C.; Tajouri, N.; Hainard, A.; Burkhard, P. R.; Lalive, P. H.; Turck, N., Exploring the human tear fluid: discovery of new biomarkers in multiple sclerosis. Proteomics Clin Appl 2014, 8, (3-4), 185-94.

212. Kim, Y. J.; Gallien, S.; El-Khoury, V.; Goswami, P.; Sertamo, K.; Schlesser, M.; Berchem, G.; Domon, B., Quantification of SAA1 and SAA2 in lung cancer plasma using the isotype-specific PRM assays. Proteomics 2015, 15, (18), 3116-25.

213. MacLean, B.; Tomazela, D. M.; Shulman, N.; Chambers, M.; Finney, G. L.; Frewen, B.; Kern, R.; Tabb, D. L.; Liebler, D. C.; MacCoss, M. J., Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics 2010, 26, (7), 966-8.

214. Garcia-Berrocoso, T.; Penalba, A.; Boada, C.; Giralt, D.; Cuadrado, E.; Colome, N.; Dayon, L.; Canals, F.; Sanchez, J. C.; Rosell, A.; Montaner, J., From brain to blood: New biomarkers for ischemic stroke prognosis. J Proteomics 2013, 94, 138-48.

215. Syrjanen, J.; Teppo, A. M.; Valtonen, V. V.; Iivanainen, M.; Maury, C. P., Acute phase response in cerebral infarction. J Clin Pathol 1989, 42, (1), 63-8.

216. Stelmasiak, J. I. a. Z., PROGNOSTIC IMPORTANCE OF MONITORING SERUM AMYLOID A PROTEIN (SAA) IN PATIENTS WITH CEREBRAL INFARCTION. Acta clin Croat 2000, 39, (139-146).

217. Azurmendi, L.; Degos, V.; Tiberti, N.; Kapandji, N.; Sanchez, P.; Sarrafzadeh, A.; Puybasset, L.; Turck, N.; Sanchez, J. C., Measuring Serum Amyloid A for Infection Prediction in Aneurysmal Subarachnoid Hemorrhage. J Proteome Res 2015, 14, (9), 3948-56.

218. Uhlar, C. M.; Whitehead, A. S., Serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem 1999, 265, (2), 501-23.

219. Malle E, D. B. F., Human serum amyloid A (SAA) protein: a prominent acute-phase reactant for clinical practice. Eur J Clin Invest. 1996, 26, (6), 427-35.

220. Gabay, C.; Kushner, I., Acute-Phase Proteins and Other Systemic Responses to Inflammation. New England Journal of Medicine 1999, 340, (6), 448-454.

221. Ahmad, M.; Graham, S. H., Inflammation after stroke: mechanisms and therapeutic approaches. Transl Stroke Res 2010, 1, (2), 74-84.

222. Wartenberg, K. E.; Schmidt, J. M.; Claassen, J.; Temes, R. E.; Frontera, J. A.; Ostapkovich, N.; Parra, A.; Connolly, E. S.; Mayer, S. A., Impact of medical complications on outcome after subarachnoid hemorrhage. Crit Care Med 2006, 34, (3), 617-23.

223. Koennecke, H.-C.; Belz, W.; Berfelde, D.; Endres, M.; Fitzek, S.; Hamilton, F.; Kreitsch, P.; Mackert, B.-M.; Nabavi, D. G.; Nolte, C. H.; Pöhls, W.; Schmehl, I.; Schmitz, B.; von Brevern, M.; Walter, G.; Heuschmann, P. U.; Investigators, F. t. B. S. R., Factors influencing in-hospital mortality and morbidity in patients treated on a stroke unit. Neurology 2011, 77, (10), 965-972.

224. Eisenhut, M., Neopterin in Diagnosis and Monitoring of Infectious Diseases. Journal of Biomarkers 2013, 2013, 10.

225. Tiberti, N.; Lejon, V.; Hainard, A.; Courtioux, B.; Robin, X.; Turck, N.; Kristensson, K.; Matovu, E.; Enyaru, J. C.; Mumba Ngoyi, D.; Krishna, S.; Bisser, S.; Ndung'u, J. M.; Buscher, P.; Sanchez, J. C., Neopterin is a cerebrospinal fluid marker for treatment outcome evaluation in

patients affected by Trypanosoma brucei gambiense sleeping sickness. PLoS Negl Trop Dis 2013, 7, (2), e2088.

226. Ulvi, H.; Emre, H.; Demir, R.; Aygul, R.; Varoğlu, A.; Kara, F., Neopterin Levels in Patients with Cerebrovascular Disease. The Eurasian Journal of Medicine 2008, 40, (2), 79-82.

227. Bustamante, A.; Vilar-Bergua, A.; Guettier, S.; Sanchez-Poblet, J.; Garcia-Berrocoso, T.; Giralt, D.; Fluri, F.; Topakian, R.; Worthmann, H.; Hug, A.; Molnar, T.; Waje-Andreassen, U.; Katan, M.; Smith, C. J.; Montaner, J., C-Reactive protein in the detection of post-stroke infections: Systematic review and individual participant data analysis. J Neurochem 2017.

228. Eklund, K. K.; Niemi, K.; Kovanen, P. T., Immune Functions of Serum Amyloid A. 2012, 32, (4), 335-348.

229. Shimizu, S., Acute phase reactant proteins in subarachnoid hemorrhages. Nosotchu 1992, 14, (3), 262-271.

230. Tsai, N.-W.; Chang, W.-N.; Shaw, C.-F.; Jan, C.-R.; Huang, C.-R.; Chen, S.-D.; Chuang, Y.-C.; Lee, L.-H.; Lu, C.-H., The value of leukocyte adhesion molecules in patients after ischemic stroke. Journal of Neurology 2009, 256, (8), 1296-1302.

231. Polin, R. S.; Bavbek, M.; Shaffrey, M. E.; Billups, K.; Bogaev, C. A.; Kassell, N. F.; Lee, K. S., Detection of soluble E-selectin, ICAM-1, VCAM-1, and L-selectin in the cerebrospinal fluid of patients after subarachnoid hemorrhage. J Neurosurg 1998, 89, (4), 559-67.

232. Lambertsen, K. L.; Biber, K.; Finsen, B., Inflammatory cytokines in experimental and human stroke. J Cereb Blood Flow Metab. 2012, 32.

233. Kim, J. S., Cytokines and adhesion molecules in stroke and related diseases. Journal of the Neurological Sciences 137, (2), 69-78.

234. Condette-Auliac, S.; Bracard, S.; Anxionnat, R.; Schmitt, E.; Lacour, J. C.; Braun, M.; Meloneto, J.; Cordebar, A.; Yin, L.; Picard, L., Vasospasm After Subarachnoid Hemorrhage. Interest in Diffusion-Weighted MR Imaging 2001, 32, (8), 1818-1824.

235. McMahon, C. J.; Hopkins, S.; Vail, A.; King, A. T.; Smith, D.; Illingworth, K. J.; Clark, S.; Rothwell, N. J.; Tyrrell, P. J., Inflammation as a predictor for delayed cerebral ischemia after aneurysmal subarachnoid haemorrhage. J Neurointerv Surg 2013, 5, (6), 512-7.

236. Lu, H.; Shi, J. X.; Chen, H. L.; Hang, C. H.; Wang, H. D.; Yin, H. X., Expression of monocyte chemoattractant protein-1 in the cerebral artery after experimental subarachnoid hemorrhage. Brain Res 2009, 1262, 73-80.

237. Ni, W.; Gu, Y. X.; Song, D. L.; Leng, B.; Li, P. L.; Mao, Y., The relationship between IL-6 in CSF and occurrence of vasospasm after subarachnoid hemorrhage. Acta Neurochir Suppl 2011, 110, (Pt 1), 203-8.

238. Aihara, Y.; Kasuya, H.; Onda, H.; Hori, T.; Takeda, J., Quantitative Analysis of Gene Expressions Related to Inflammation in Canine Spastic Artery After Subarachnoid Hemorrhage. Stroke 2001, 32, (1), 212-217.

239. Fassbender, K.; Hodapp, B.; Rossol, S.; Bertsch, T.; Schmeck, J.; Schütt, S.; Fritzinger, M.; Horn, P.; Vajkoczy, P.; Kreisel, S.; Brunner, J.; Schmiedek, P.; Hennerici, M., Inflammatory cytokines in subarachnoid haemorrhage: association with abnormal blood flow velocities in basal cerebral arteries. Journal of Neurology, Neurosurgery & Psychiatry 2001, 70, (4), 534-537.

240. Gaetani, P.; Tartara, F.; Pignattit, P.; Tancioni, F.; Rodriguez, R.; Baena, B.; De Benedettit, F., Cisternal CSF levels of cytokines after subarachnoid hemorrhage. Neurological Research 1998, 20, (4), 337-342.

List of Publications from Leire Azurmendi

- **Azurmendi L**, Degos V, Tiberti N, Kapandji N, Sanchez-Pena P, Sarrafzadeh A, Puybasset L, Turck N, Sanchez JC, (2015) "***Neopterin plasma concentrations in patients with aneurysmal subarachnoid hemorrhage: correlation with infection and long-term outcome."*** J Neurosurg: 124(5): 1287-99

- **Azurmendi L**, Degos V, Tiberti N, Kapandji N, Sanchez P, Sarrafzadeh A, Puybasset L, Turck N, Sanchez J-C, (2015) "***Measuring Serum Amyloid A for Infection Prediction in Aneurysmal Subarachnoid Hemorrhage."*** Journal of Proteome Research 14: 3948-3956

- **Azurmendi L,** Degos V, Tiberti N, Kapandji N, Sanchez P, Sarrafzadeh A, Puybasset L, Turck N, Sanchez J-C, (2017) ***"Infection prediction for aneurysmal subarachnoid hemorrhage patients at hospital admission: combined panel of serum amyloid A and clinical parameters"*** – Journal of Transl Sci 3(3): 1-5

- **Azurmendi L,** Lapierre-Fetaud V*, Schneider J, Montaner J, *Katan M, *Sanchez JC, (2017)***"Proteomic discovery and verification of Serum Amyloid A, a predictor marker of patients at risk of post-stroke infection: a pilot study"** Clin Proteom 14:27

- **Azurmendi L*,** Lagerstedt L*, Sanchez JC. ***"Applications of amine-reactive tandem mass tags (TMT) in human neuroproteomics"*** Submitted to chapter book Neuromethods Series-Springer