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TGF beta expression in plasma and cerebral spinal fluid following aneurismal subarachnoid haemorrhage (aSAH): temporal profile during early and delayed ischemic injury.

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Subarachnoid Haemorrhage

In a report published in 1810, Blackall described anatomic features of a ruptured basilar artery attributing it to the cause of sudden death in the Swedish crown prince Charles August, an event that led to a new royal dynasty existing to this day. Samuel Wilks, a British physician who worked in Guy's hospital in London, further characterized clinical and pathological features of sanguineous meningeal effusion in four case series at autopsy and coined the term spontaneous subarachnoid hemorrhage in 1859. Later in the beginning of 1900, Symonds wrote as in the picture and coined the term subarachnoid haemorrhage (SAH) in 1924 (1).

Section of Neurology.

President—Dr. JAMES S. COLLIER.

Spontaneous Sub-arachnoid Hæmorrhage.

By C. P. SYMONDS, M.D.

THE title of my paper calls in the first instance for a word of explanation. From a study of the literature it seems that the term "spontaneous" in this connexion has acquired for some persons a mystical significance, and is conferred by them upon those cases of sub-arachnoid hæmorrhage for which no apparent cause is to be found either at the bedside or after death. *Omne ignotum pro magnifico!* Let us admit the existence of many cases, the origin of which remains obscure, and suffer the impatience of our present ignorance without seeking refuge behind a formula.

The term "spontaneous," as I interpret it here, covers all cases of sub-arachnoid hæmorrhage of origin other than traumatic. The word is used improperly, but I must refer you as precedent for the distinction to one of the earliest papers upon the subject published by Sir Samuel Wilks in 1859 and entitled "Sanguineous Meningeal Effusion (Apoplexy): Spontaneous and from Injury."[1]

Blood appearing in the sub-arachnoid space may obtain entrance to it by various channels. It may be derived from the rupture of vessels lying within the sub-arachnoid space itself. It may find its way into the sub-arachnoid space from hæmorrhage into the subdural cavity (as commonly occurs in traumatic lesions of the veins passing from the cerebral cortex into the great sinuses).

Or again, blood derived from the rupture of a vessel within the nervous substance is not infrequently discharged into the sub-arachnoid space either by breaking through the pia mater if the hæmorrhage be superficial, or, more usually, since cerebral hæmorrhage is, as a rule, deeply situated, the effusion may burst into one of the ventricles and make its way into the general sub-arachnoid cavity by the channels which connect this with the ventricular system.

Of these three sources of sub-arachnoid hæmorrhage I propose only to discuss the first and third, since, so far as I am aware, subdural hæmorrhage does not occur except as the result of injury, and is therefore excluded from present consideration.

The clinical evidence of sub-arachnoid hæmorrhage may be considered under two heads, first that derived from the examination of the cerebro-spinal fluid, second that which may be obtained by observation of the history, symptoms and course of the illness.

The changes which occur in the cerebro-spinal fluid in this condition were fully described by Froin in his thesis of 1904, and there is little to add to his original observations.

Shortly after the onset of the hæmorrhage the fluid obtained by lumbar puncture appears on withdrawal to be mixed with blood, and is usually under increased pressure.

[1] *Guy's Hosp. Rep.*, 1859, 3rd ser., v, p. 119.

Symonds CP. *Symonds CP (1924).* "Spontaneous Sub-arachnoid Hæmorrhage". *Proceedings of the Royal Society of Medicine.* ***17*** *(Neurol Sect): 39–52.*

SAH is a severe emergency due to the extravasations of blood in the subarachnoid space. The leading cause of non-traumatic SAH is an intracranial aneurysm rupture (aSAH), accounting for more than 80% of SAH cases and for 5% of total strokes. The early mortality rate after aSAH remains high at 40%, and 10-20% of patients never reach medical attention or die during transport. The general estimated incidence is 8–10 cases per 100.000 inhabitants per year. Risk factors include hypertension, smoking, alcohol abuse, female gender (3:2), the use of sympathomimetic drugs (e.g., cocaine) and genetic syndromes, as autosomal dominant polycystic kidney, Black and Hispanic ethnicity. Most aneurysms occur on the Circle of Willis close to bifurcations. The most commons sites for ruptures are the posterior communicating artery/internal carotid artery take off and the anterior cerebral artery (2).

Figure 1 shows the anatomical space interested from the bleeding. Figure. 2 shows the arteries of the Willis polygon where arterial aneurysms are mainly located. Figures 3 a-b-c shows CT scan appearance of patients with SAH with contrast and with 3D reconstruction.

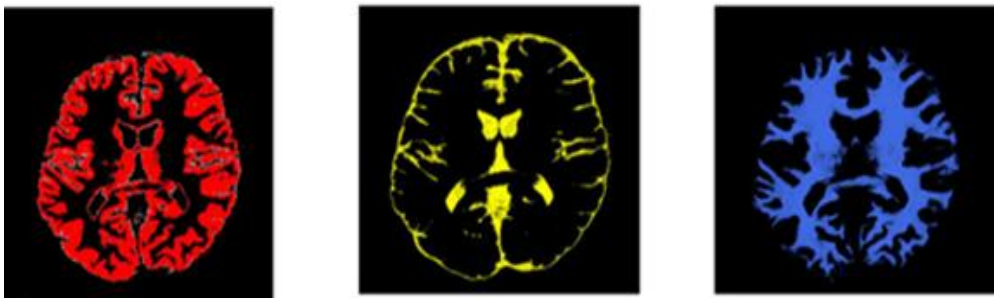


Fig. 1. Red: grey matter; yellow is the CSF and ventricles that represent the subarachnoid space, Bleu: white matter. This picture has been produced in Cambridge UK by the author using parametric maps of colour coregistrered on MRI scans of one male healthy subject.

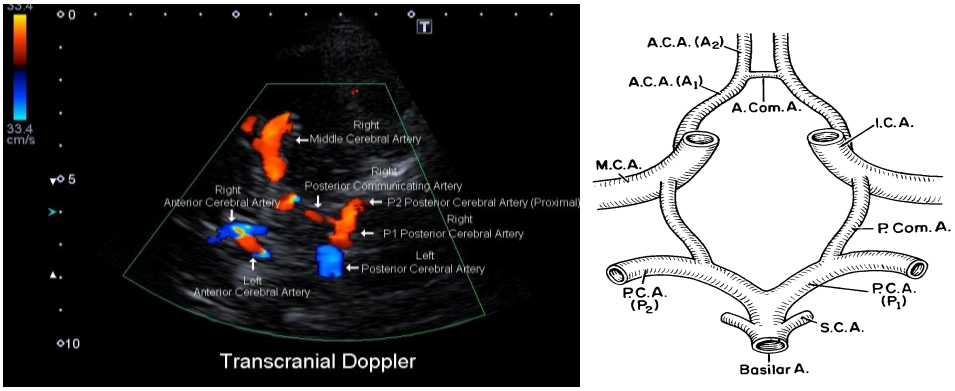


Fig 2. The Polygon of Willis. A.C.A (1 and 2) is the anterior cerebral artery, A. com A is the anterior communicating artery, and P.C.A (1 and 2) is the posterior cerebral artery. M.C. A is the middle cerebral artery, I.C. A is the internal carotid artery, Basilar Artery; S.C.A is the superior cerebellar artery. Ecocolor Doppler appearance of polygon of Willis.

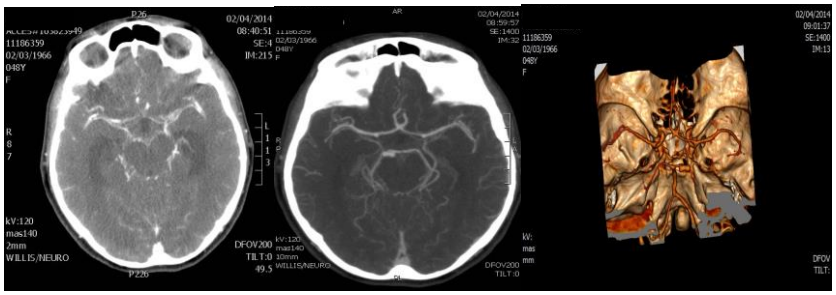


Fig 3. Right posterior cerebral artery aneurism. This picture shows the CT appearance of the first patient included into the study.

1. Principles of treatment, clinical and radiological indicators of outcome.

Aneurismal SAH is a heterogeneous illness with different clinical exordia and outcomes. Patients typically complaint of a sudden onset or “thunder clap” headache, described as “the worst in my life”. Associated features range from nausea and vomiting, neck stiffness, deteriorating state of consciousness and, in severe cases, cardiac arrest. The first line investigation is the computed tomography (CT) scan with high sensitivity (95-100%). CT angiogram has a negative predictive value of 82-96% for detect aneurysms and is most sensitive for those major than 4 mm in size. If further investigation is warranted, digital subtraction angiography (DSA) is the gold standard for the cause of SAH diagnosing (2, 3).

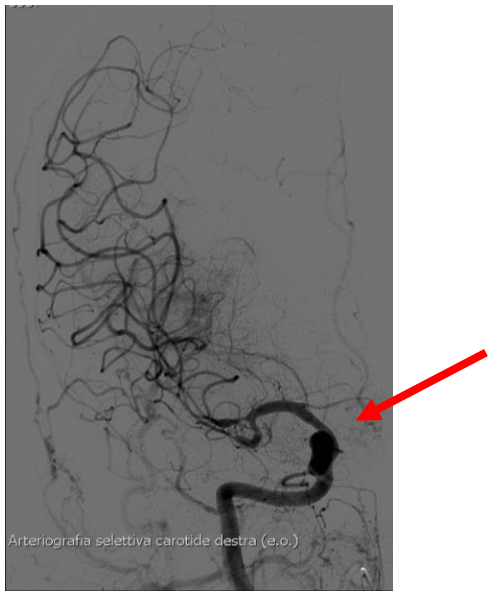


Fig. 4 this picture shows DSA of patient suffering from aneurysm of ICA, red arrow.

The time course of the disease divides in different phases contributing significantly to the overall outcome: the extent of the initial haemorrhage, the intervention to treat the ruptured aneurysm with the occurrence of early hydrocephalus and the general medical management following the bleeding.

The extent of the initial haemorrhage and the clinical scenario demands the initial stabilization of the patient with assessment of clinical characteristics: i.e. sudden coma or seizures. Later we will describe the consensual ongoing metabolic and physiopathological alterations responsible for the early and late cerebral ischemia associated to the clinical scenario. There are two clinical and one radiological score to define the severity of illness *per se* at exordium: the Hunt Hess scale, The WFNS (world federation of Neurologic Surgeons) and the modified Fisher Scale (summarized together in Tab. 1) (4).

Grade	Fisher Modified	Hunt and Hesse	WFNS
I	minimal or thin SAH without IVH	No symptoms or mild headache and neck stiffness	GCS 15, no motor deficit
II	I plus IVH	Moderate or severe I symptoms ± cranial nerve palsy	GCS 14-13 without motor deficit
III	Thick SAH Without IVH	Mild focal deficit, lethargy or confusion	GCS 14-13 with motor deficit
IV	III plus IVH	Stupor ± hemiparesis	GCS 7-12 ± motor deficit
V	-	Deep coma ± extensor posturing	GCS 3-6 ± motor deficit

Tab. 1. This table summarize the three scores together. GCS, Glasgow coma scale; IVH, intraventricular haemorrhage.

The ruptured aneurysm must be treated as soon as possible to prevent the rebleeding with the surgical clipping or endovascular coiling. In light of recent findings from literature (ISAT – The International Subarachnoid haemorrhage Trial) (5) clipping is

reserved for aneurysms that are unsuitable for coiling *i.e.* wide neck or MCA locations. Early hydrocephalus develops frequently and placement of an external ventricular drain (EVD) results in rapid clinical improvement in one third of patients, allowing intracranial pressure control and measure.

Initial management focus on cardiorespiratory stabilization to maintain cerebral perfusion and oxygenation by securing airways, controlling ventilation and with careful blood pressure management (6, 7, 8).

Once the aneurysm is secured, the medical management takes place mainly in neurocritical care units aiming at optimizing systemic physiology and preventing or treating delayed neurological deficit (described later). In fact, at the time of the bleeding and later in the course of the disease, systemic medical complications occur and negatively affect overall morbidity and mortality. Among those, cardiac stunning and neurogenic pulmonary oedema are due to a massive catecholamine discharge. Fever without infections, severe electrolytes disturbances mainly hyponatremia with two syndromes CSW (cerebral salt wasting syndrome) and SIADH (syndrome of inappropriate secretion of antidiuretic hormone) are frequent in SAH patients (8, 9, 10). Derangements of cerebral autoregulation have been widely described in literature. In our Neurointensive Care Unit (NICU), we measure cerebral pressure reactivity with a dedicated software (11).

Anaemia occurs in a large number of patients within 3-4 days following SAH, more common in females and is associated with high rates of DINDS, mortality and worse neurological outcome in survivors (7).

The table below shows different percentages of systemic and intracerebral complications following SAH (7).

Intracranial and systemic complications	
Delayed hydrocephalus	25-30%
Fever	70%
Cardiopulmonary complications	25-30%
Anaemia	20-40%
Fluid and electrolyte imbalances	30-50%
Seizures clinical / Non convulsive sub-clinical seizures	25% overall /10-20%

2. SAH is an ischemic disease. Delayed Ischemic Neurological Deficits (DINDs): focus on vasospasm (VS) and inflammation.

Despite starting with a sudden bleeding, aSAH generates ischemic lesions. The abrupt discharge of blood into the basal cisterns raises the intracranial pressure and reduces cerebral blood flow. Later in the course of the disease, many complications affect brain parenchyma and contribute to DINDS.

The most feared intracranial complications after SAH are seizures, hydrocephalus with intracranial hypertension, derangements in cerebral autoregulation and, above all, VS (12).
Hydrocephalus is present in 30% of patients within 3 days with higher risk in poor grade and patients with large ventricular clot. Hydrocephalus requires the insertion of an extra ventricular drain (EVD) insertion. Seizures are common but frequently underdiagnosed and patients with poor grade SAH, deteriorating, or fail to improve, need to have an electroencephalogram to exclude non-convulsive status epilepticus (13). The main cause of DINDS is VS that has been the focus of most of the studies in the last 20 years (12).
It is widely recognised that VS and DINDS are not synonymous.

The Neurocritical care society guidelines recommend the following definition:

1. DINDS is a neurological deterioration related to ischemia (unrelated to the treatment of aneurysm) that persist for more than one hour and has no longer cause (hydrocephalus, seizures or metabolic).
2. VS is the arterial narrowing demonstrated angiographically or with Doppler ultrasonography, with corresponding clinical symptoms and signs arteries.

VS occurs among day 4 and day 21 after bleeding. In about 60% of patients surviving the initial haemorrhage, VS happens but, in some cases, angiographically detection of VS does not correspond to DINDS. Fifteen percent of patients affected from VS die or have devastating neurological damage. Risk factors associated to VS are smoke, poor grade SAH, large blood clot with IVH. The clinical symptoms are the decrease in the level of consciousness and focal signs such as aphasia and hemiparesis, that may be reversible or progress to cerebral infarction.

Pathophysiological changes in arterial wall consist in thickening, impaired vascular relaxation, impaired pressure reactivity and autoregulation. The lumen narrowing and reduced cerebral blood flow (CBF) with ischemia in affected areas follow. The initial trigger for arterial narrowing is the contact between the oxyhemoglobin that accumulates after the bleeding with the ab-luminal side of the vessels.

One of the leading theories on the genesis of this prolonged contraction of smooth muscle cells of blood vessels involves the release of cytosolic calcium. The breakdown of blood in subarachnoid space release oxyhemoglobin that inhibits the ATP-dependent calcium pump. Calcium levels in CSF are low in patients with VS. Much like calcium other compound affect vasoconstriction: endothelin 1 (described later) and nitrite/ate levels. Products of arachidonic acid oxidation, F2-isoprostanes, correlate with poor outcome and induce vasoconstriction.

Indeed VS, has radiological and clinical manifestations. Interestingly the merely vessel narrowing is not the only responsible for the late cerebral ischemia. Both radiological (i.e. the strumental confirmation of arterial narrowing without clinical remarks) and clinical VS may be part of a larger cellular and molecular process that affects the entire neurovascular unit.

Diagnosis entails primarily of a high index of suspicion with daily monitoring, in addition of neurological clinical examination. Diagnostic tools currently available and routinely used in our Unit are reported in appendix 1.

Ability to prevent or treat cerebral VS could improve significantly survival and neurological outcome. Current treatment methods include primary medical therapy and in selected patients endovascular methods (Fig.5) but these measures (alone or in combination) can been ineffective (15, 16).

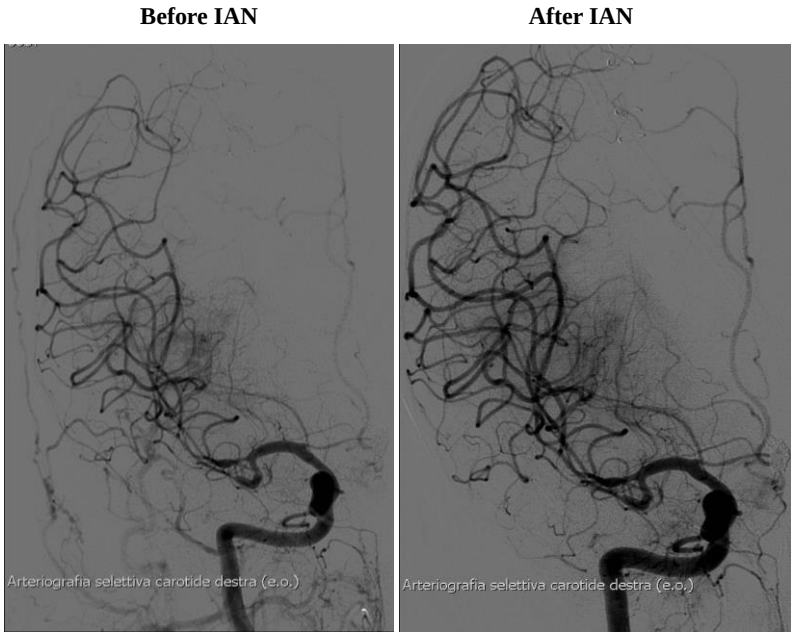


Fig. 5 Angiographic demonstration of VS and reversion with intra-arterial calcium antagonist nimodipine (IAN). The solely vasodilatation has no proven benefit on tissue viability and neurological outcome (15).

To date, *Caveats* from clinical literature highlight the following points:

1. Nimodipine (calcium channel blocker) does not affect vessel caliber but it is associated to a better neurological outcome. Nimodipine is a dihydropyridine calcium channel antagonist. In the British Nimodipine trial there was a 34% and 40% reduction in cerebral infarction and poor outcome in patients as compared to placebo. Nimodipine is administered orally 60 mg 4 hourly starting at the time of diagnosis until day 21 with caution to avoid hypotension (17).
2. The aim of blood pressure control is to maintain cerebral perfusion pressure (CPP) that is equal to the difference between the mean arterial pressure (MAP) and the intracranial pressure (ICP). In VS, the increased blood pressure is to overcome the arterial narrowing. Blood pressure and cardiac output management aim to euvolemia with arterial blood pressure augmentation with simultaneous assessment of neurological status when possible (7).
3. Clazosentan (ET-1 Receptor antagonist) significantly improve vessel caliber but has no proven benefit on neurological outcome. The study CONSCIOUS-1 did not show reduction in the number of cerebral ischemic

events. Phase II trials (CONSCIOUS II and III) did not show effect on morbidity and mortality (18).

4. Fisher (1981) showed that the thickness of subarachnoid blood clot correlates with the likelihood to VS development (19).
5. Endovascular treatment options are angioplasty and intra-arterial vasodilator therapy, alone or in combination. Prophylactic use is not recommended. If maximal medical therapy has failed, this can be considered (16).
6. Small diameters arterioles play important role in the autoregulation of cerebral blood flow. Constriction of small arteries can contribute to ischemia and remain undetectable to angiography (20, 21).
7. Data on Antiplatelet prophylaxis are inconclusive. Antiplatelet agents are indicated only when a stent is deployed in the treatment of a ruptured aneurysm (7).
8. The current evidence does not support the use of antifibrinolytic drugs in the treatment of people with aneurysmal subarachnoid haemorrhage, even in those who have concomitant treatment strategies to prevent cerebral ischaemia. Results on short-term treatment are promising, but not conclusive
9. To date, ability of consistent prediction of VS onset and severity is still poor, no effective preventive therapy is available. Clinically reliable biomarker for cerebral vasospasm and DINDS has yet to be identified.

3. Moving towards early cerebral ischemia and early brain injury (EBI)

Recent research focuses on the aberrant changes that happen soon after the initial haemorrhage. The term EBI describes the immediate injury to the brain after SAH, before onset of delayed vasospasm and it is the damage sustained within the first 72 hours SAH. EBI derives from alterations in CBF autoregulation, disrupted cerebral metabolism, and impaired permeability of the blood brain barrier, discharge of inflammatory cytokines, leukocytes, and pro-thrombotic pathways.

It is likely that EBI may have a central role in the instigation of DINDS and poor outcome overall.

As said above, most of the research and therapies are directed towards reducing the incidence of VS. To date, because clinical trials have not delivered a definitive treatment for preventing or reducing brain injury after SAH, there is a paradigm shift towards EBI. Recent studies have already focused on the use of therapeutic agents able to lessen EBI. Examples include statins (22) which can attenuate the caspase-dependent apoptosis pathway; melatonin, a neuron-hormone with antioxidant effects that mitigates cerebral oedema in experimental animal models and hyperbaric oxygen.

EBI starts at the time of aneurysm rupture. Haemorrhage into the subarachnoid space initiates a complex cascade of deleterious mechanisms that cause damage to the central nervous system. The sudden discharge of blood in basal cisterns and ventricles raises intracranial pressure and impairs cerebral perfusion pressure - CPP and CBF. Intracranial pressure - ICP increases and reduces CBF by compression of the vasculature, increases the total resistance within the vessel circuit and interferes with cerebral autoregulation. Blood and its degradation products within the subarachnoid space act as spasmogens on the vessels, and promote the formation of delayed vasospasm as described above. Disruption of cerebral autoregulation and the rapid breakdown of the blood brain barrier end in vasogenic oedema and the initiation of an inflammatory response via the release of vasoactive substances like endothelin-1 and matrix metalloproteinases in response to inflammatory cytokines. These inflammatory cytokines also coordinate a massive recruitment of neutrophils and macrophages into the subarachnoid space and surrounding affected tissue, perpetuating EBI. Endothelial cells interactions, inflammation, oxidative stress and spreading depolarization participate to the genesis of EBI (23, 24). The following scheme reports events of EBI (modified from ref 25).

EBI	TIME LINE OF ISCHEMIA RISK	
1st hour ↑ICP↓ CPP ↓CBF Altered autoregulation • ↑ CSF Glutamate and Brain water content • Vasoconstriction ↓endothelial function, ↑ permeability, ↓ perfusion Activation of cell death mediators ↑ Oxidative stress ↓ No ↑ Platelet activation ↑ ET-1 ↑ Proinflammatory cytokines	**24 hrs.** ICP stabilizes at a level > baseline CPP recovers Autoregulation remains altered CSF glutamate remains ↑ Hydrocephalus, Hyponatraemia, Vasodilation, Vasodilation, Permeability remains ↑ Perfusion recovers Progression of cellular death ↑ Oxidative stress, No, Platelet activation, ET-1 ↑ Proinflammatory cytokines	**72 hrs.** ICP, CPP, and FSC revert to baseline condition Autoregulation remains altered, CSF glutamate remains ↑ Hydrocephalus, Hyponatraemia Vasospasm, Endothelial cell degeneration Permeability remains ↑ Apoptosis Perfusion recovers Progression of cellular death ↑ Oxidative stress, No, Platelet activation, ET-1 ↑ Proinflammatory cytokines

Clinical features and biomarkers of EBI that could potentially have beneficial prognostic and therapeutic implications are currently under investigation.

4. Biomarkers in SAH, review of literature

In the construction of the design of this study, starting from our publications in the field of aSAH considerations and clinical experience, we moved through a wide literature research. We started applying sequentially Medline filters as shown in picture below (Fig 5) and then we merged our results with the ones derived from other Authors analogue studies of systematic review. Large volumes of literature exist on biomarkers (BMs) analysis within the context of different human biological samples and SAH. Distinct techniques and theoretical perspectives have been used to study biomarkers at different historical moments. Each method evolved from the theoretical understanding of an underlying pathophysiological mechanism in the development of VS or in EBI. Most of the studies agree on the concept that more mechanisms and molecules sustain brain damage in SAH and that only panels of BM gave best results as compared to a single BM. Pubmed query results with the numbers of papers reported are summarized below in the figure 5.

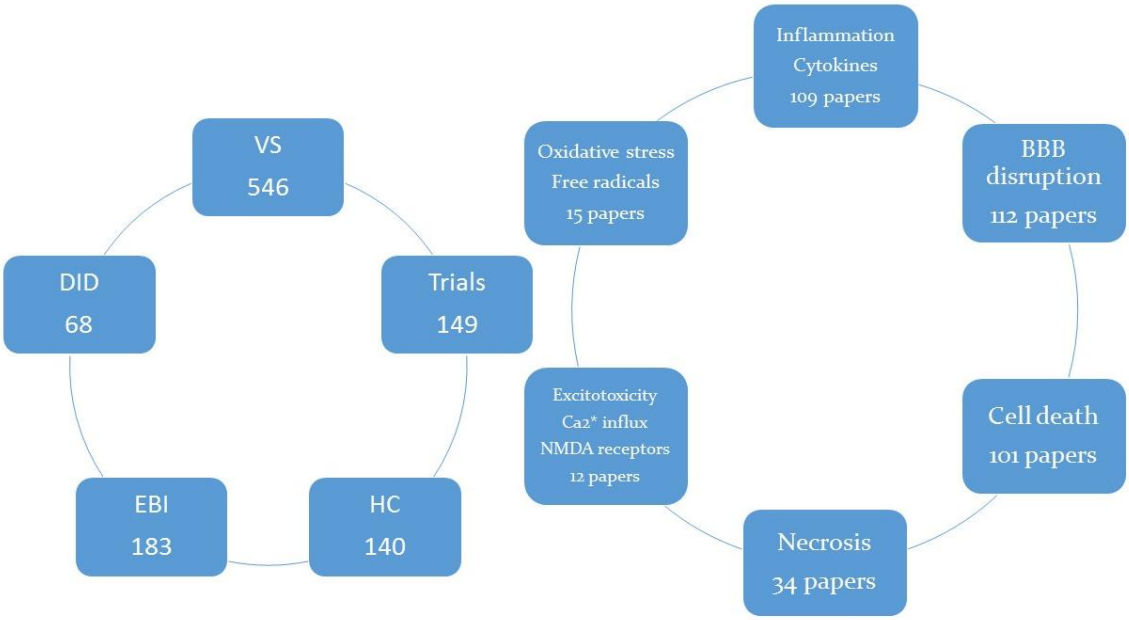


Fig 5. On the left: results for 1st query, SAH and VS, EBI, VS, Hydrocephalous (HC) and clinical trials. On the right side, the picture shows the number of papers found in literature affording the 2nd query based on the mechanism of action implicated in early and late brain damage following SAH.

We started narrowing the query and we identified different categories of biomarkers according to the pathophysiological mechanism of action: inflammation, cell damage, change in cellular metabolism and vascular tone, micro particles derived biomarkers. We decided not to include Genotyping polymorphisms works. Then, we went on tapering our query and

considered the two main sides of the topic in brain pathology: inflammation and neurodegeneration.

We then dichotomized the research focusing on two points:

a. Markers of inflammation currently described in SAH and effectors of vasoconstriction
b. Markers of other pathological mechanisms associated in humans and animals to brain damage, excitotoxicity sustained by necrosis and apoptosis

a. Markers of inflammation currently described in SAH and effectors of vasoconstriction.

Brain is an immunologically privileged organ with the exception of microglia that take part to immune response. Inflammation is widely recognized as part of the pathophysiology of VS. In fact, some Authors (26) have shown that systemic inflammatory activation is common after SAH even in the absence of infection with aSAH triggering an immune activation sufficient to induce a systemic inflammatory response syndrome, SIRS. Higher burden of SIRS in the initial four days independently predicted symptomatic vasospasm and was associated to worse outcome. In this review attempt we then moved forward, beyond the context of SAH and sought for the activation of inflammatory biomarkers in forms of VS in meningitis, cerebral malaria and

brain injury (27, 28). There is evidence for common pathways of inflammation involving the below mentioned biomarkers and is widely demonstrated that there is a calcium dependent and independent VS. In fact, haemoglobin causes contraction of major cerebral arteries reducing neuronal nitric oxide synthase in the *adventitia* of the cerebral arteries. The reduction of NO availability causes prolonged contraction regardless to the concentration of intracellular calcium.

The output of the literature review on the main inflammatory mediators that interact with cerebral vasculature is the follow:

1. Nitric oxide (NO) reduction determines the reduction of the vasodilatory capacity by the reduction of the guanylate cyclase. NO is inactivated by reaction with the superoxide dismutase resulting in the formation of peroxy-nitrite. Reactive oxygen (anions) species are released in different conditions as brain injury, seizures, ischemia and reperfusion, and meningitis. Inflammatory cytokines as TNF down regulate NO. Statins are associated to reduction of VS in humans and animal models. Statins act upregulating the expression of endothelial NO synthase.
2. Endothelin 1 (ET-1) is the most potent vasoconstrictor in blood vessels, it is produced in cerebral endothelium and

mediates vasoconstriction by the pathway of endothelin-A (ETA) in vascular smooth muscle cells. ETA stimulates calcium concentration and vessel contraction. ET-1 secretion is stimulated by TGF beta, TNF alpha, IL-1 and haemoglobin. ET-1 is inhibited by NO and dilator prostanoids as via increase of cGMP and natriuretic peptides by an increase of cAMP levels.

3. Inhibition of potassium channels. 20-hydroxyecosatetraenoid acid from arachidonic acid via P-450 enzyme is a potent vasoconstrictor that inhibits the potassium channels. In some experimental models, Nicorandil (2-nicotinamidoethyl-nitrate ester), an ATP-sensitive potassium channel opener with nitric oxide (NO) like activity, is used as a treatment for angina and acute heart failure and it has been associated to a reduction of VS due to the reactivation of potassium channels.

4. Interleukins 1 and 6 are expressed in CSF of patients with SAH. IL-1 beta is correlated to VS development trough G-proteins with 3 mechanisms. A calcium dependent VS via myosin contraction, activation of protein kinase C that sustain the myosin light chain prolonged contraction not sustained by calcium and a third mechanism of contraction of myosin light chain sustained by the activation of myosin light chain phosphatase trough

activation of rho-kinase receptors. Some studies on the drug hydroxyfasudil (a rho-kinase inhibitor) demonstrated reversion of VS in some case series.

5. Platelet derived growth factor (PDGF) is another mediator implied in the non-calcium dependent VS by inhibition of tyrosine kinase.

6. TNF-α is a proinflammatory cytokine associated to oxidative stress, cell death and recruitment of inflammatory mediators. Early expression of TNF–alpha in some studies was associated to worse neurological outcome. TNF-alpha increases the expression of vascular endothelium adhesion molecules as VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intracellular adhesion molecule-1) that promotes the uptake of circulating leukocytes. Microglia is activated from this inflammatory mediators and lead to production of proteases, free radicals and complement that potentiate the inflammatory response.

7. Acute phase protein, such as C reactive protein (CRP), has been described in SAH and increased expression in CSF was associated to early occurrence of VS (29, 30, 31, 32, 33).

b. Markers of other pathological mechanisms associated in humans and animals to brain damage

and excitotoxicity sustained by necrosis and apoptosis.

Apoptosis and necrosis are two process of cell death distinguishable but not mutually exclusive. In both mechanisms, a step rise in cytosolic concentration of calcium take place. In general, apoptosis is an energy-dependent process whereas necrosis is not.

Neurons survival depends on adequate oxygen and substrates supply as glucose lactate and ketones. Even a brief ischemic insult to the brain may trigger complex cellular events that lead to progressive apoptotic and necrotic cell death. Necrosis starts with an abrupt cessation of oxygen and glucose supply with a massive failure to ATP production. Cell breakdown is due to acute depolarization, loss of neurons excitability and massive efflux of glutamate. As consequence, a massive afflux of calcium into the cells follows and the NMDA receptors for glutamate are activated. Calcium afflux into mitochondria destroys DNA and lead to massive energy failure. Apoptosis is a selective death directed by different genes following DNA damage or when energy supply is low. When energy and flow restore, the delayed death occurs even in absence of structural damage.

Other Cell death mechanisms such as autophagy, endoplasmic reticulum stress, microcirculatory dysfunction and cortical spreading depression injury contribute to neuronal damage.

The common initial phenomenon after the aneurysm rupture is acute CBF reduction or arrest, resulting in a state of transient global brain ischaemia that can itself be lethal. In cases in which patients survive such events, secondary ischaemic insult may appear due to changes in the blood-brain barrier (BBB), and it may progress to the point of causing generalised cerebral oedema and/or apoptosis. Cerebral oedema contributes to yet another increase in ICP, resulting in further reduction of the CBF. The mechanism of BBB impairment is unclear, but apoptosis is one possible cause. In SAH, if the initial bleed is severe enough to prevent blood flow to the brain as in a global stroke, it is unlikely that the brain tissue would survive. In this case massive necrosis is the main determinant of EBI and eventually brain death. Otherwise, in case of survival, apoptosis starts at the time of EBI. The progression from EBI to DINDS is not fully elucidated in literature and is part of the scope of this thesis.

In a case series from Sima R and co-workers a panel of neurodegeneration biomarkers was investigated in CSF and blood of patients with SAH. Most of the markers were quantified using the enzyme linked immunoassay (ELISA) with a

methodology similar to the one we adopted in the present study (36).

The second output of our Literature review summarize the follows biomarkers:

1. S100β, astroglia enriched protein rises in several conditions affecting brain metabolism.
2. NSE, neuron enriched specific enolase is a glycolytic enzyme released from neurons that has been widely described as a marker of severe neuron loss following cardiac arrest, stroke, encephalitis and neurodegenerative stress in numerous studies.
3. CCSntf and CCSctf, calpain-derived α-spectrin N and C terminal fragments are frequently expressed in necrosis and have been demonstrated to be upregulated following SAH.
4. Ubiquitin C-terminal hydrolase L1- UCHL1 is a proteolytically stable protein released from neurons and neuro endocrine cells. It is a marker of neuron loss, it indicates damage mainly to dendritic cells. Elevated levels of UCHL-1 on day two following SAH are described.

5. Calpain and caspase are cysteine proteases activated in response to complex apoptotic signalling via proteins such as Bcl-2 (B cell lymphoma 2) and Bcl-xL. This molecules act controlling the mitochondrial membrane with dissipation of H+ and uncoupling the respiratory chain.
6. Akt (protein kinase B), a serine/ threonine kinase, is one of the key anti-apoptotic signalling molecules downstream of phosphoinositide 3-kinase (PI3K) in EBI after SAH.
7. Mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, have all been studied in EBI. JNK and p38 are activated in response to inflammatory cytokines and cellular stress, up-regulating apoptotic cascades.
8. The tumour suppressor gene, p53, also regulates apoptosis. In EBI after SAH, anti-apoptotic therapies have reported to ameliorate outcomes by targeting the MAPK pathway activating p53 and hypoxia inducible factor-1 (HIF-1) target genes by hyperbaric oxygen (37, 38, 39, 40, 41, 42).

Given the clinical nature of the study, a part from biological markers associated to worse neurological outcome and brain ischemia, we reviewed the clinical condition that are known to be associated to brain damage in SAH. Fever, hyperglycaemia, hypoglycaemia, severe anaemia, hypotension, intracranial hypertension and seizures were also included into the design and analysis of this study (7).

5. The neurovascular unit. The role of microglia and TGF-β

In light of our review, we moved a way forward. As a final point to design the study, we constricted our main question and focused on the anatomical space involved in CBF and brain metabolism interface as a functional space.

As widely known, cerebral hemodynamic and brain metabolism match. Regional blood flow is proportional to the demand for oxygen and glucose that are the main energy substrate for neurons. Intracranial hypertension reduces cerebral blood flow and decreases energy metabolism. The cycle of glutamine and glutamate sustain the 80% of brain energy in astrocytes and the levels of glutamate and glutamine in CSF following SAH correlates to the incidence of VS. From the histopathology point of view, the link between cerebral hemodynamic and brain metabolism derangements is the neurovascular unit, which constitutes the BBB.

The vascular components of the neurovascular unit are the endothelial cells, the contractile cells surrounding the endothelial cells (myocytes in the arterioles and pericytes in the capillaries), and the basal lamina of the vessel. Endothelial cells display selective transport by transcytosis. During inflammation, this mechanism fails disrupted and there are alterations in

permeability of endothelial tight junctions and formation of oedema.

The nervous system specific components of the neurovascular unit are the astrocytes, which project throughout the vessels, and the neurons, peripherally located and in contact with the astrocytes. Between the vascular and nervous system specific components, there is the Virchow-Robin space, which is an inflection of the leptomeninges over the pial vessels where these vessels enter the brain parenchyma.

This anatomy explains why lesions of the neurovascular unit can release molecules and cells that are detectable by laboratory tests into both the cerebrospinal fluid (CSF) and the bloodstream.

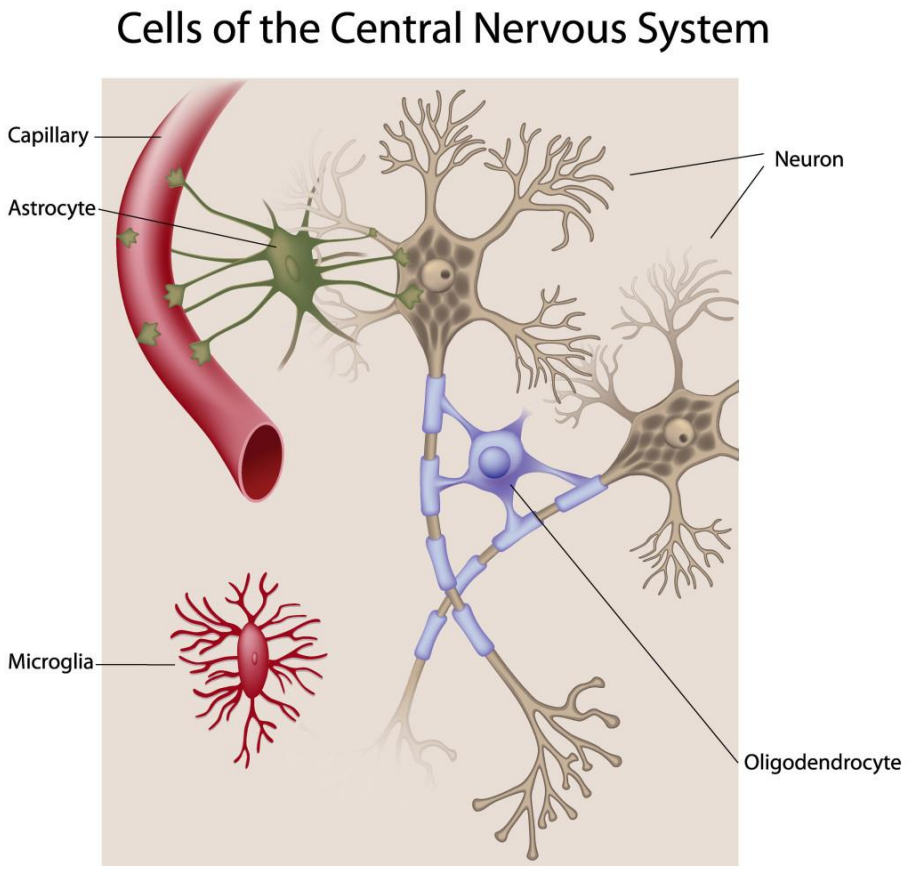
In 1846, Virchow first recognized the existence of a fragile, non-nervous, interstitial component made up of stellate or spindle-shaped cells, morphologically distinct from neurons, which he named neuroglia, or "nerve glue." Later in the twentieth century, distinct cell types divided into three broad groups of glial cells classified this interstitial component:

1. True glial cells or macroglia, such as astrocytes and oligodendrocytes, of ectodermal origin, the stem cell of which is the spongioblast;
2. Ependymal cells, also of ectodermal origin and sharing the same stem cell as true glia.
3. Microglia (MG), that origins for mesoderm. MG is small, relatively sparse cells with elongated nuclei and little

cytoplasm, difficult to observe in microscopy. MG can be stained and demonstrated by light microscopy using Hortega's silver carbonate method. No comparable technique exists for their ultrastructural demonstration.

Glial cells differ from neurons in that they possess no synaptic contacts and retain the ability to divide throughout life, particularly in response to injury. In this study, we decided to focus on microglia starting from these considerations

a. MG is located in the normal brain in a resting state and it is of monocyte origin, and fill a role as immune effector cells in the central nervous system. Almost 239 genes and 8 microRNAs are uniquely or highly expressed in microglia as compared to myeloid and other immune cells and constitute the molecular signature of microglia itself non influenced by bloodstream molecules. Once activated, MG first proliferate, then extend the length of their nuclei, then either form aggregates around tissue necrosis or form aggregates around the cell bodies of dying neurons. There is a resident pool of microglia that is separate from peripheral myeloid cells that infiltrate the nervous system. Recent studies confirm that resident microglia represent a unique, indigenous cell population in the brain.



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b. Studies on the role of MG in brain damage already exist. Proliferation and activation of microglia occur after haemorrhagic stroke, SAH and intracerebral haemorrhage. In intracerebral haemorrhage models, resident cerebral macrophage or microglia can consume heme released from damaged red blood cells and degrade it via heme oxygenase. Other studies showed that MG can

regulate blood clearance also in SAH by heme oxygenase-1.

In a very interesting paper, A Hanafy and co-workers showed the critical role that microglia plays in facilitating vasospasm and neuronal apoptosis. In this study, the Author depleted microglia using clodronate liposomes and showed that in both early and late phases of SAH, microglia was necessary for vasospasm. Moreover, in the early phase of SAH (within post-operative day -POD 7) neuronal apoptosis was largely microglial-dependent. By POD 15 (late phase SAH), neuronal apoptosis was microglial-independent (44).

c. Some Authors demonstrated the requirement of TGF-β for the in vitro development of microglia and that there is absence of microglia in CNS of TGF-β1 deficient mice. A unique microglial signature exists and is dependent on TGF-β signalling. It has been reported that in the experimental autoimmune encephalomyelitis (EAE) model, infiltrating monocytes do not contribute to the residual microglial pool and that microglia can be distinguished from monocytes. Recruited monocytes do not acquire the microglia signature and then microglia and recruited monocytes maintain their own molecular signature during neuroinflammation. In a number of disease, such as trauma, MG is stimulated and migrate to the area of injury, where they phagocytose debris. Loss of

microglia is documented in mice deficient for TGF-β1 in the CNS. Other studies showed that TGF-β modulates microglial phenotype and promotes recovery after ICH. However, the neurotoxic versus protective properties of MG and the role of TGF-β are not fully elucidated across the broad spectrum of brain pathologies, mainly over the time course of different diseases. The identification of a molecular TGF-β1 dependent microglial signature may provide new insights into microglial biology and the possibility of targeting microglia for the treatment or prevention of early and late injury in SAH (43, 44, 45, 46, 47).

We have chosen to analyse the expression of TGF-β in CSF and plasma of patients suffering from SAH as a possible biomarker involved in pathophysiology of EBI and DINDS because we focused on anatomical and functional district, the MG. We aimed to describe a temporal profile of TGF-β expression during early and delayed ischemic injury in our cohort of patients with SAH.

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Chapter II

Transforming Growth Factor beta (TGF-β)

Chapter II Transforming Growth Factor beta (TGF-β)

1. TGF-β structure, secretion and mechanism of action
2. TGF-β in Neurological and Neurosurgical pathologies
3. TGF-β in SAH
4. References

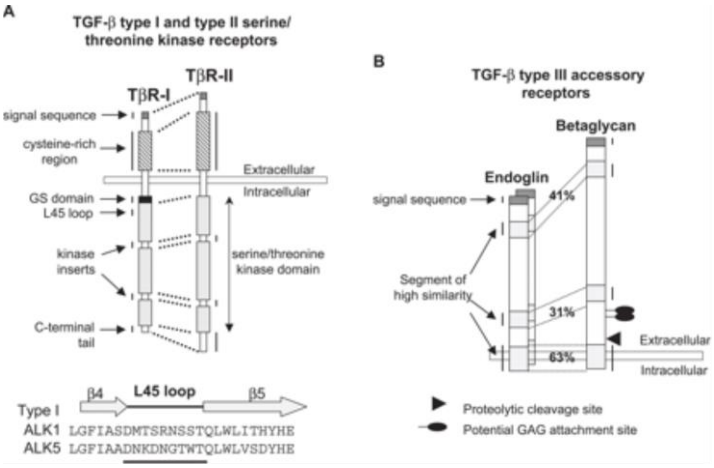
1. TGF-β structure, secretion and mechanism of action

Transforming growth factor beta is a highly researched cytokine in the fields of cancer, autoimmune diseases and infectious disease. TGF-β has diverse and multiple roles throughout the body with many effects on cell proliferation, differentiation, morphogenesis, tissue homeostasis and regeneration, and the severe diseases that result from their malfunctions (1,2).

TGF-β is a multifunctional cytokine belonging to the transforming growth factor superfamily that includes four different isoforms (TGF-β 1 to 4), and many other signalling proteins produced by all white blood cell lineages. Each isoform is synthesized as a precursor that includes a signal peptide to direct TGF-βs to the endoplasmic reticulum, a large N-terminal portion called the latency-associated peptide, and a short C-terminal fragment, which is the mature cytokine. The predominant isoform expressed in the immune system is TGF-β1, the mature form of which exists as a latent, biologically inactive 25 kDa protein, composed of two identical 12.5 kDa subunits. TGF-β has pleiotropic effects on adaptive immunity, especially in the regulation of effector and regulatory CD4+ T cell responses. T cells themselves are an important source of TGF-β for regulating adaptive immune responses.

TGFβ1 promote epithelial and mesenchymal cell proliferation and differentiation. Mice lacking this isoform either die embryonically or, if surviving to birth, develop multi-organ inflammation and die by 3–4 weeks of age. This highlights a crucial role for TGF-β in dampening self-harmful inflammatory responses. However, as described in many papers in literature, TGF-β also has important functions in promoting inflammatory responses.

Mechanism of action starts with signalling in cells via binding to the TGF-β receptor complex, a tetrameric structure composed of two type I TGF-β receptors (TGF-βRI) and two type II TGF-β receptors (TGF-βRII).



From: TGF-β receptor function in the endothelium Cardiovasc Res. 2005;65(3):599-608

The assembly of the TGF-β receptor complex triggers recruitment of the intracellular receptor Smad (R-Smad) proteins

Smad2 and Smad3 to the cytoplasmic domain of activated TGF-βRI, which directly phosphorylates Smad2/3. Once phosphorylated, Smad2/3 forms a trimeric structure with Smad4, which translocate to the nucleus to activate or repress gene expression. The activation of different downstream substrates and regulatory proteins induces the transcription of different target genes that function in differentiation, chemotaxis, proliferation, and activation of many immune cells.

Schematic role of TGF-β in different cellular populations

Cells	Chemotaxis	Cytotox.	Survival	Proliferation	Effector	Ag pre.	Mat/diff
Dendritic						↓	↓
Eosinoph	↑						
T cells			↑	↓	↓		↓
B cells			↓	↓			
Mon/Macro	↑					↓	
Nk	↓	↓					↑
Mast	↑						

2. TGF-β in Neurological and Neurosurgical pathologies

Field of application of TGF-β varies according to its putative multiple actions. Consequently, studies point on TGF-β role in promoting inflammation and fibrosis, others in enhancing apoptosis and neurodegenerative process (Alzheimer's and Huntington's disease) and psychiatric disorders. The role of TGF-β in the genesis of primary tumours is not part of this work.

Most of available research on TGF-β focuses on brain and intraventricular Haemorrhage (IVH) in new-borns. IVH is associated with damage to periventricular white matter and the damage is exacerbated by the development of hydrocephalus; combinations of pressure, distortion, ischaemia, inflammation, and free radical-mediated injury add further damage. The harm to white matter accounts for the high frequency of cerebral palsy in these infants. The identification of mechanisms and mediators of hydrocephalus and white matter damage is leading to the development of new treatments to prevent permanent hydrocephalus and its neurological complications and shunt dependence.

Recent experimental studies have suggested that acute parenchymal compression and ischemic damage, and increased parenchymal and perivascular deposition of extracellular matrix proteins--probably due, at least partly, to upregulation of TGF-β

are further important contributors to the development of the hydrocephalus.

Heep et al. investigated the role of TGF-β in Neonatal Posthemorrhagic Hydrocephalus (PHH), They found that TGFβ1 was higher in patients with PHH than in patients with hydrocephalus from other reasons and that there was a correlation between persistent TGFβ1 and the occurrence of white matter injury in patients with PHH. They concluded that CSF malabsorption might contribute to the pathogenesis of PHH through the TGFβ1 signalling cascade, which in turn regulates collagen synthesis and promotes cell proliferation and that the persistent elevation of TGF-β may be of prognostic value to indicate severity of brain injury in patients with PHH (3).

Interestingly, TGF-β could already be a biomarker ready for routine clinical use to determine which infants with PHH have obstructive hydrocephalus and may benefit from a ventriculostomy procedure. Lipina et al. published the sensitivity and specificity of a particular TGFβ1 cut-off point for this decision. This group has proven statistically relevant probability in diagnosis of hyporesorptive hydrocephalus based on TGFβ1 levels in CSF. Value exceeding 3,296 pg/ml means 81,3% probability of present hypo-resorption. Success rate of ventriculostomy in patients with MRI-verified obstruction and TGFβ1 levels lower than 3,296 pg/ml was 100% in this series.

Studies on Intracerebral haemorrhage (ICH) investigated the specific response and activation of microglia and attempted to describe a temporal profiling during the acute and resolution phase following haemorrhage.

The unique microglia signature indicates that microglia and blood derived macrophages are independent.

Interestingly in ICH, the predominant expression of TGF-β was associated to reduction in microglia expression of inflammatory cytokines. Patients who expressed increased levels of TGF-β had better neurological outcome and that microglial TGBβ1 transformed from a pro inflammatory to a reparative function in ICH (4). This beneficial effect is not contradictory if we put these results in the context of a different pathology as PHH (and eventually SAH). If TGF-β promotes reparative fibrosis, this effect into cerebral ventricles is detrimental as intraventricular fibrosis is associated to development of hydrocephalus (see later studies on SAH) but into the parenchyma the effect of TGF-β on scar and tissue healing, might be beneficial. Many other experimental animal models of ICH explored how the modulation of microglia/macrophage polarization ameliorate intracerebral haemorrhage-induced inflammatory response (5,6).

Studies on cerebral cavernous malformations are not conclusive.

Brain arteriovenous malformation (BAVM), a rare but important cause of intracranial haemorrhage, have increased angiogenesis and inflammation as key components of the nidus of abnormal vessels and stroma that form the resected surgical specimen.

Accordingly, both vascular endothelial growth factor (VEGF) and transforming growth factor-β have been implicated in the pathology of BAVM for their proangiogenic and vascular-regulating roles (7).

Studies on the expression of TGF-β in ischemic stroke also exist. Haemorrhagic transformation (HT) is a major complication of ischemic stroke that worsens outcomes and increases mortality. Disruption of the blood-brain barrier is a central feature of HT pathogenesis, and leukocytes may contribute to this process. Similarly to studies on ICH, the over expression of TGF-β in neonatal focal arterial stroke, showed that microglial cells were activated and this was associated to the secondary haemorrhage prevention. Microglial depletion by intracerebral injection of liposome-encapsulated clodronate 5 significantly reduced vessel coverage and triggered haemorrhages in injured regions 24 h after ischemic stroke further demonstrating that TGFβ1 signalling in microglia protects from HT (8). Interestingly, Authors determined that ischemic strokes that develop HT had differences in RNA expression in transforming growth factor-β signalling in blood within 3 hours of stroke onset prior to treatment with thrombolytic therapy. RNA prediction of HT in stroke was demonstrated in this paper (9).

Studies on traumatic brain injury showed that the Transforming growth factor-β 1 signalling regulates neuroinflammation and apoptosis thus contributing to the secondary brain injury. The activation of TGFβ1 in TBI correlated with the induction of free

radical generating enzyme NADPH oxidase 1 (NOX1). Further inhibition of TGF-β type I receptor TGF-βRI or TGFβ1 and transfection of TGFβ1 siRNA and TGF-β antagonist Smad7, diminished TGFβ1-induced inflammation and apoptosis (10).

3. TGF-β in SAH

Studies on TGF-β in SAH exist and focus mainly on Hydrocephalus.

TGF-β promotes fibrosis. Fibrotic tissue remodelling is the final common pathological outcome of many chronic inflammatory and infectious diseases. Tissue repair and regeneration are critical biological processes that are fundamental to the survival of all living organisms. Although the synthesis of extracellular matrix components like collagen is an indispensable and, typically, reversible part of all wound-healing responses, normal tissue repair can evolve into a progressively irreversible fibrotic response if the tissue injury is severe or repetitive or if the wound-healing response itself becomes dysregulated. Wound-healing responses are tightly regulated. When the wound-healing response is well organized and controlled, the inflammatory response resolves quickly, and normal tissue architecture is restored. However, if the wound-healing response is chronic or becomes dysregulated, it can lead to the development of pathological fibrosis or scarring, impairing normal tissue function.

Hydrocephalus develops from fibrosing arachnoiditis, meningeal fibrosis and sub-ependymal gliosis, which impairs flow and resorption of cerebrospinal fluid (11). Authors have already

described that TGF-β is released by platelets into the subarachnoid space and it is associated to long-term shunt dependency.

Douglas and co-workers collected samples of CSF from 20 patients following SAH and acute hydrocephalous. CSF samples were taken from an external ventricular drainage every second day following admission to Hospital. CSF samples were drawn randomly in some patients and not systematically. Samples were centrifuged at 720 g for ten minutes and supernatant was aliquoted and stored at -70 degrees. The CSF concentrations of TGFβ1 and 2 (latent and active form) were determined by antibody sandwich enzyme linked immunosorbent assay (ELISA). Because of the small number of samples, the results were pooled and divided in 4 time points summarizing groups at time 1-5, 6-9, 10-14 and 15-19 days post haemorrhage group means compared. Means of total TGF-β peaked on day 1-5, thereafter levels decreased but still remained higher than in control group (patients with hydrocephalous for reasons other than SAH). As in previous studies, these authors determined (to disclose if the raised levels of total TGF-β originated from either the primary haemorrhage and/or rebleeding) levels of albumin in CSF. The latter showed a decline over days, indicating that no further bleeding was responsible for the sustained levels of TGF-β in CSF. Main result of this study was that at all acute point the TGF-

β levels were higher in patients that developed chronic hydrocephalus (12).

Two other papers focused the role of TGF-β in the genesis of hydrocephalus following SAH, and proposed a novel treatment for it. Decorin, a small leucine-rich chondroitin-dermatan sulphate proteoglycan expressed by neurons and astrocytes in the central nervous system, is both anti-fibrotic and anti-inflammatory and attenuates the formation and partial dissolution of established and chronic scar. This small leucine-rich proteoglycan is a pan-receptor tyrosine kinase (RTK) antagonist acting and is the Transforming growth factor-β antagonist. In this study, after inducing subarachnoid haemorrhage, rats were treated with humans recombinant Decorin. This treatment resulted in reduction of subarachnoid fibrosis mediated and alleviated chronic hydrocephalus via inhibiting TGF-β1/Smad/CTGF pathway. Moreover, Decorin significantly decreased myelin damage in the caudal internal capsule and prevented caudal periventricular white matter oedema and astrogliosis attenuating long-term neurocognitive defects after SAH (13).

Other Authors demonstrated that inhibition of TGF-β attenuates brain injury and neurological deficits in a rat model of germinal matrix haemorrhage (GMH). GMH increased the level of TGFβ1 and activated the TGF-β pathway in brain tissue. Inhibition of GMH-induced activation of the TGF-β pathway led to significant

cognitive and motor improvement and attenuation of GMH induced brain atrophy and development of hydrocephalus (14).

Similarly, other Authors disclosed the Thrombin-induced TGFβ1 pathways the cause of communicating hydrocephalus post SAH.

In their experiment they infused CSF with thrombin (TH), resulting in proinflammatory and proliferative responses in rat meninges of SAH. The effect of TH was completely blocked by a TGFβ1 inhibitor, SB-431542, suggesting that TH-stimulated proliferation of meninges is through the TGFβ1 signalling pathway. The cascade of TGFβ1-Smad3 was significantly upregulated by TH, which, in turn, stimulated the proliferation of subarachnoid meninges. TH-induced overexpression of TGFβ1 and activation of its downstream factors (15).

Finally, in reviewing literature, it appears that there is no precise phenotype marker for diagnosis of intracranial aneurysm (IA) and the genetic predisposition to aneurysm development is still under investigation. Environmental risk factors that associate with IA can result in modifying the effect of inherited genetic factors and thereby increase the susceptibility to SAH.

Authors showed that proinflammatory cytokine TNF-α and IFNG sustain the development of IA through stochastic regulation of IL-10 and TGFβ1 by comorbid factors. Therefore, genetic liability to inflammatory response caused by polymorphisms in cytokine genes might be the common denominator in the development of

aneurysm and complications associated with rupture. The study suggests that the chronic exposure to inflammatory response mediated by genetic variants in pro-inflammatory cytokines TNF-α and IFNG could be a primary event, while stochastic regulation of IL10 and TGFβ1 response mediated by comorbid factors such as hypertension may augment the pathogenesis of IA through vascular matrix degradation (16).

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Scope of the thesis

Biomarkers might facilitate early detection of impending ischemia, stratify categories of patients at risk of DINDs and improve the monitoring of the response to treatments.

In this work, we measured the concentration of the TGF β in plasma and CSF of patients with SAH and correlate its temporal profile across the population and within single patients to clinical characteristic, evidence of early and late ischemia and outcome.

Chapter III

Materials and Methods

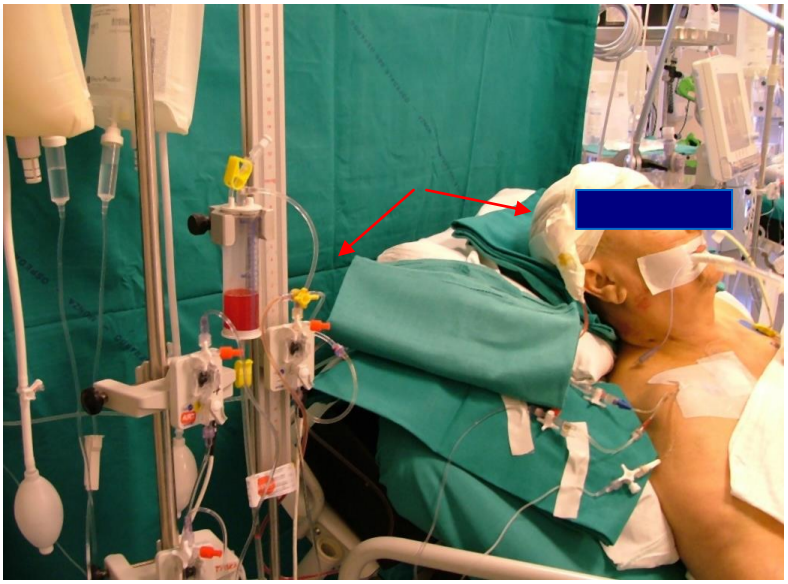
Chapter III Materials and Methods

1. Design, Population and setting
2. Clinical and laboratory records, strumental and radiological examinations
3. Sampling and quantification of TGF-β
4. Outcome and Quantification of ischemic burden on CT scan with the ASPECTS

1. Design, Population and setting

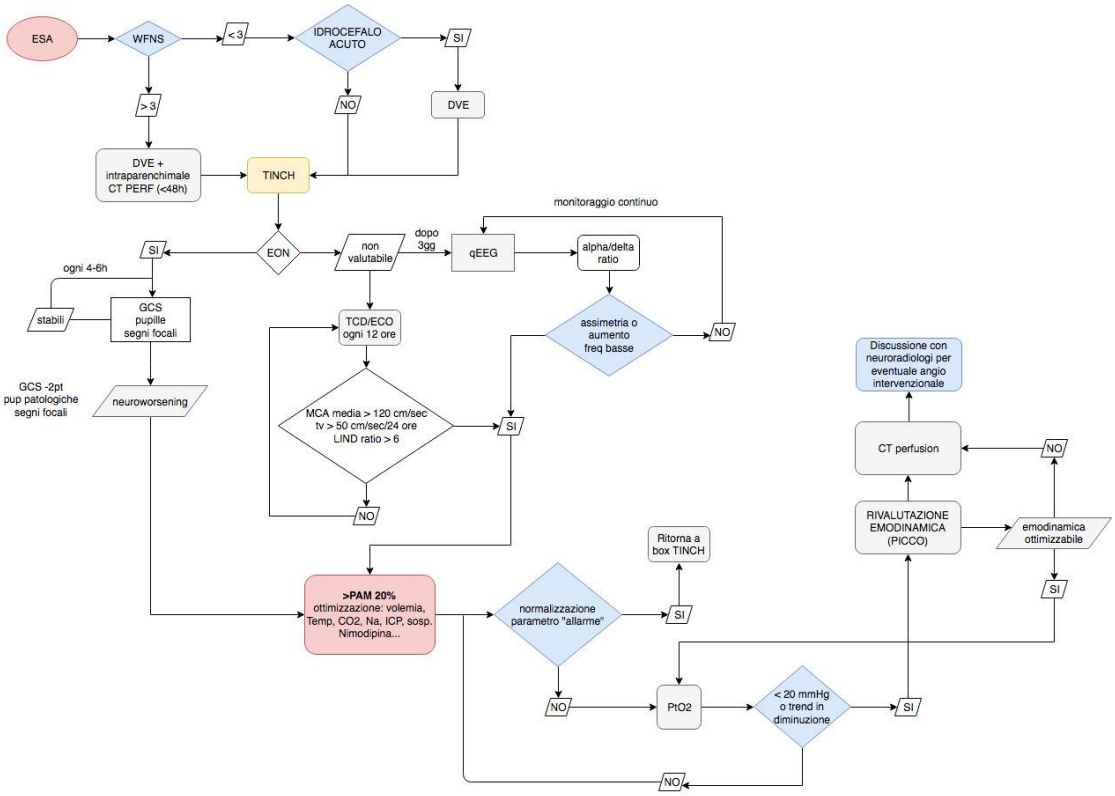
Fifty-two patients admitted to our Neurosurgical Intensive Care Unit from 2014 to 2017 were included in the study. Inclusion criteria were: age > 18, presence of aneurysm rupture with SAH documented on radiological investigation and placement of a ventriculostomy the allowed CSF drainage (EDV-extra ventricular drainage) as showed in picture below.

Figure 9. Patients n.5 mechanically ventilated on day 3 following SAH, red arrows point the extra cranial emergence of the EVD underneath the dressing and the reservoir were CSF is collected to measure the hourly drain



Aneurysm was secured within 24 hours from bleeding with either coiling or clipping. Nimodipine was administered for 21 days but discontinued in some patients were the impact on blood pressure was considered not acceptable on clinical basis. EVD was positioned if patients had GCS < 8 or significant Hydrocephalus. Patients were treated as per standard international procedures for the management of SAH.

In our NICU, succeeding initial stabilization, the algorithm of management is the follow:



Once VS takes place, induction of hypertension with high MAP to counteract vessels narrowing, mandatory for patients with DINDS unless cardiac status exclude it. Cerebral angioplasty and/or selective intra-arterial vasodilator therapy is considered in our NICU in patients with symptomatic cerebral vasospasm, particularly those who are not rapidly responding to hypertensive therapy. Once DINDS is likely, other neuroprotective strategies include sedation, hypothermia and cerebral perfusion pressure optimization (optimal CPP). Derangements in cerebrovascular autoregulation, that is the intrinsic capacity of the cerebral arteries to maintain constant CBF despite changes in CPP, are involved in the development of DINDS following aSAH. High ICP and persistent auto regulatory failure after SAH are independently associated with the occurrence of delayed cerebral infarction and may be an important cofactor in addition to VS itself. Assessment of the status of cerebrovascular autoregulation, measuring the capability of vessels of auto regulate with the pressure reactivity index (PRx), identify patients with an increased risk of DID. In our NICU we measure PRx with a custom made software (11).

2. Clinical and laboratory records, strumental and radiological examinations

Demographic data, Clinical records, strumental and radiological examinations, laboratory data, neurological and radiological scores (HH, Fisher and WFNS) were recorded. Clinical, laboratory data e continuous systemic and haemodynamic parameters were monitored with ours computed based system innovian Chart assist Draeger® (fig 10). Any drug including sedation, corticosteroids and non-steroidal anti-inflammatory drugs were systematically recorded.

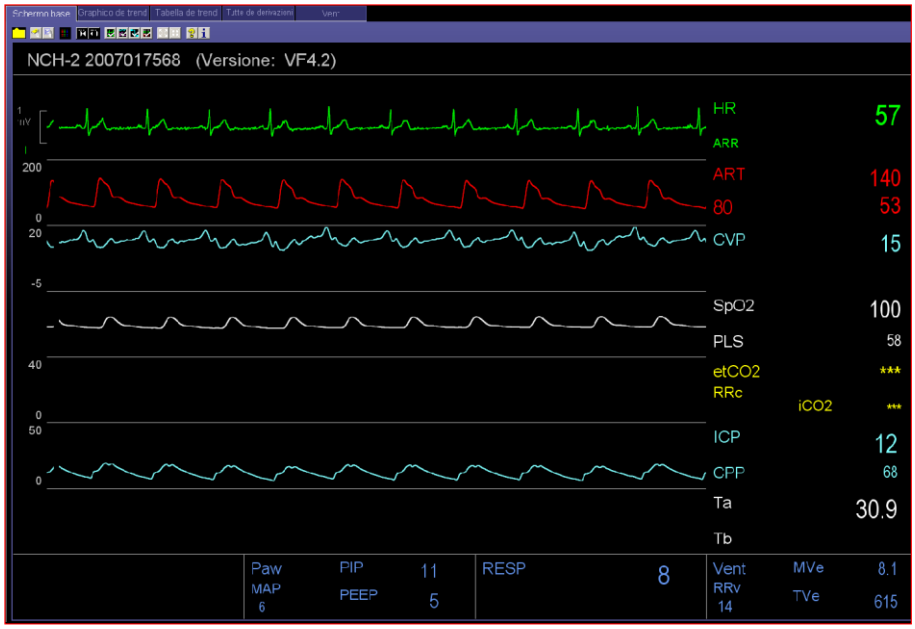


Figure 10. HR - heart rate, ART – invasive arterial blood pressure continuous monitoring, CVP- central venous pressure, SpO2 –

arterial blood saturation with pulsoxymeter, ICP- intracranial pressure, CPP- cerebral perfusion pressure.

Any drugs, fluid input and output, microbiological test results were also recorded, especially with a focus on the occurrence of infections that might affect the expected results. No antiepileptic drugs were administered unless clinical evidence of seizures was noted of continuous EEG documented NCCSE as showed in the picture 11. In our NICU we monitor c and q EEG that is quantitative graphical representation of the EEG raw using the quantitative Fourier algorithm.

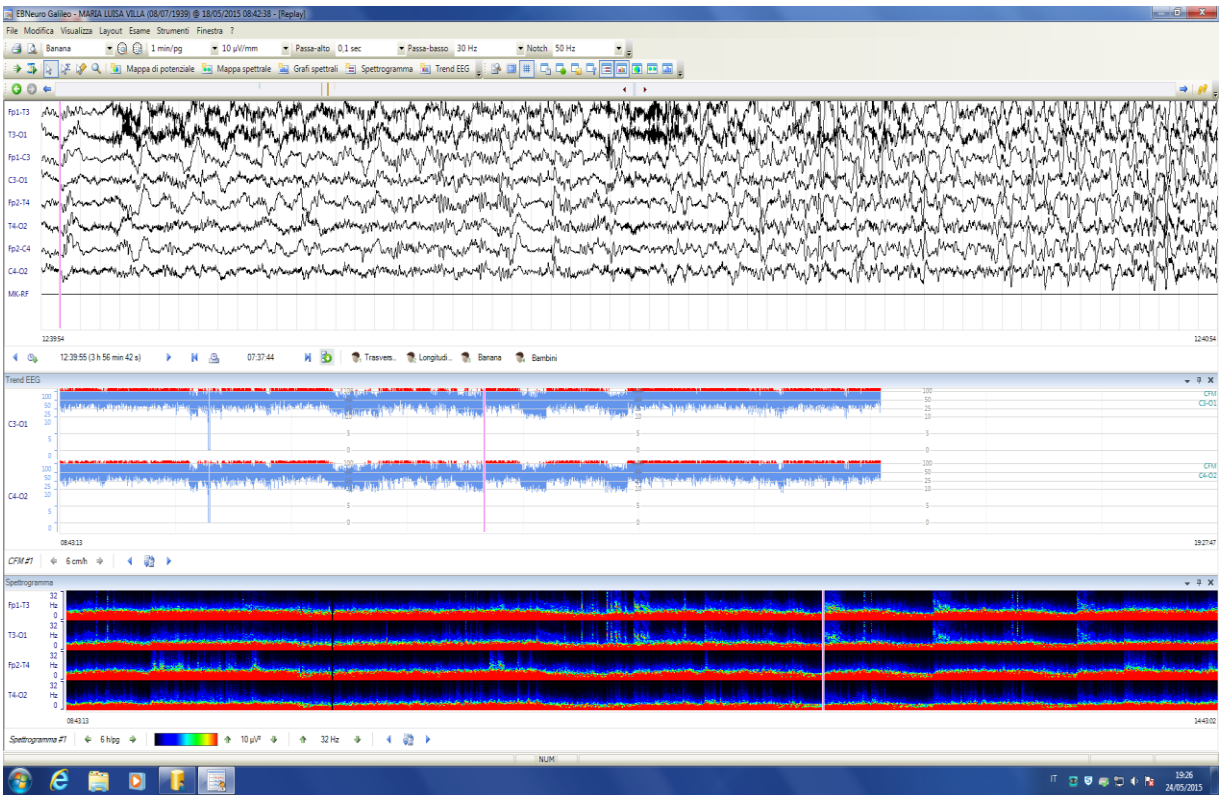
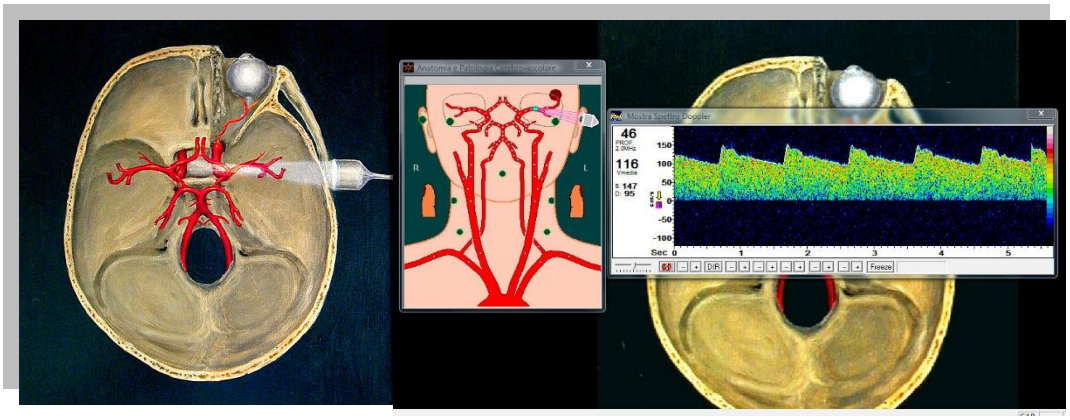


Fig 11. Raw (cEEG) and quantitative (qEEG) monitoring

Symptomatic VS is diagnosed as the development of new focal neurological signs, deterioration in level of consciousness, or both, when the cause was felt to be ischemia attributable to vasospasm after other possible causes of worsening (for example, hydrocephalus, seizures, metabolic derangement, infection, or over-sedation) had been excluded. DINDS was defined as symptomatic vasospasm or the appearance of new infarction on CT or MR when the cause is felt to be attributable to vasospasm.

Strumental detection of EBI and late DINDS was performed with c-qEEG, TCD, CT perfusion imaging and then DSA, if it was present clinical and TCD evidence of VS. we transferred patients to DSA facilities is blood pressure augmentation with IV fluids, increased cardiac output and vasopressors failed to counteract VS and revert clinical symptoms. Intra-arterial nimodipine boluses or continuous infusion was performed in some patients.

Transcranial Doppler (TCD) measure the cerebral blood flow velocities increase as sign of cerebral vessels narrowing. TCD is a simple and non-invasive bedside screening tool to detect the vasospasm but its sensitivity and specificity in identifying VS is good for MCA and inferior for other vessels. TCD vasospasm is defined as a mean flow velocity in any vessel >120 cm/sec. Perfusion imaging (perfusion CT) measures CBF and MTT (mean transit time) with diagnostic accuracy Threshold values of 35 mL/100 g/min for CBF and 5.5-second MTT suggestive for DINDS DSA is still the gold-standard technique but it is invasive and time-consuming. Angiographic vasospasm is defined as moderate-to-severe arterial narrowing on digital subtraction angiography. We move to this further step, after perfusion CT, only when an endovascular treatment is planned. Picture of severe VS reverted with intra-arterial nimodipine and ballooning is shown in the picture 5 above in chapter 1.



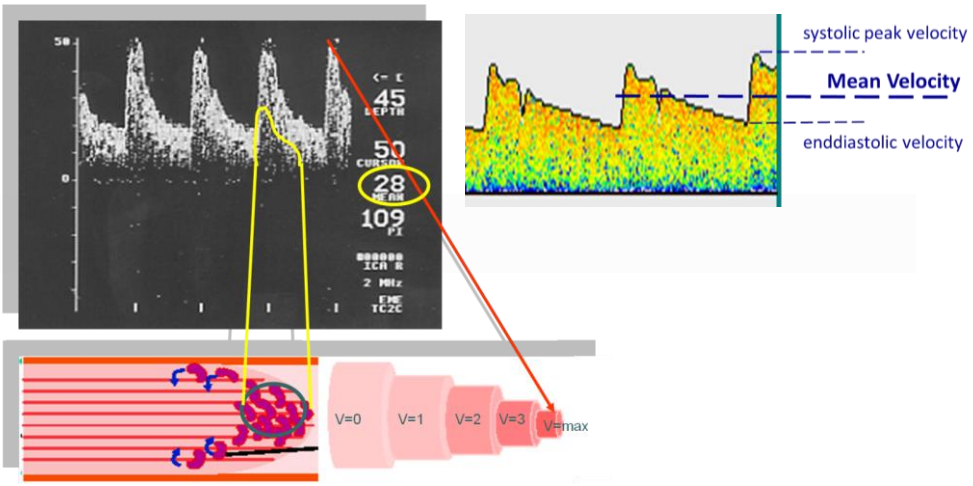
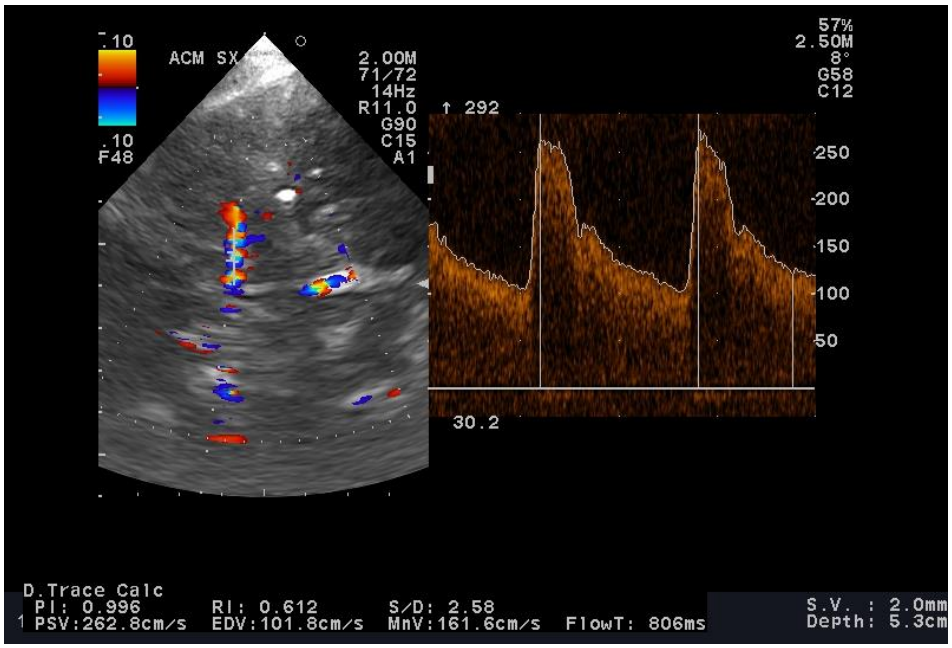


Fig 12 TCD technology


ACM SX
2.00M
71/72
14Hz
R11.0
G90
C15
A1
57%
2.50M
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250
200
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30.2
D.Trace Calc
PI: 0.996
RI: 0.612
S/D: 2.58
1 PSV:262.8cm/s
EDV:101.8cm/s
MnV:161.6cm/s
FlowT: 806ms
S.V. : 2.0mm
Depth: 5.3cm

Fig 13.a

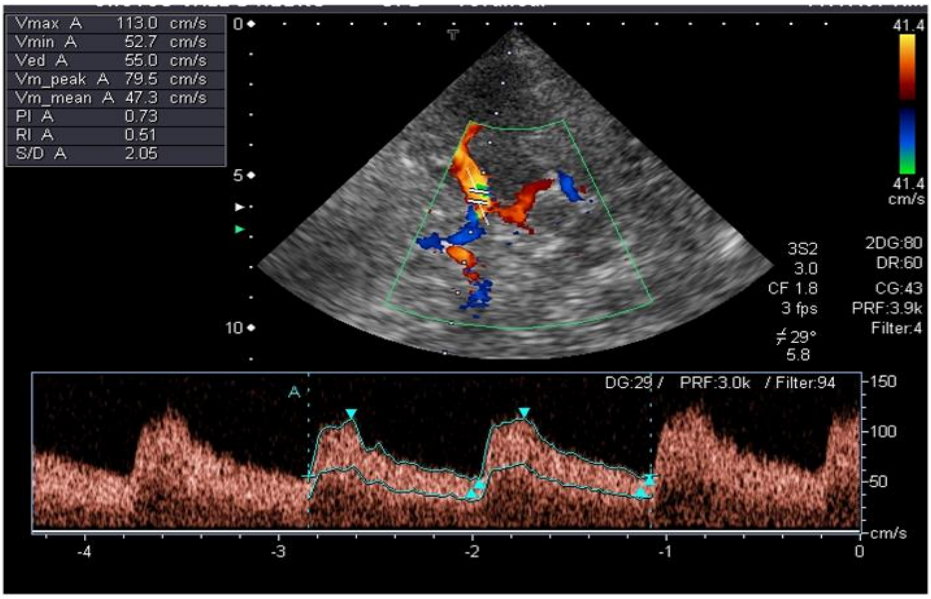
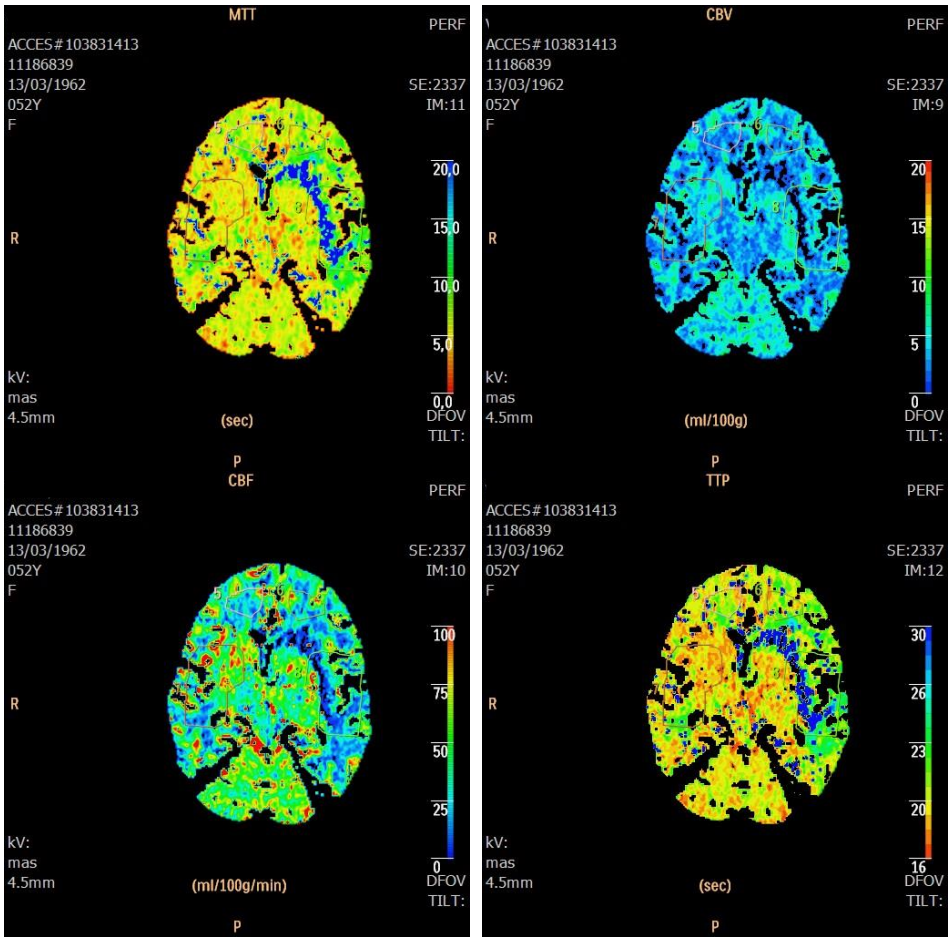
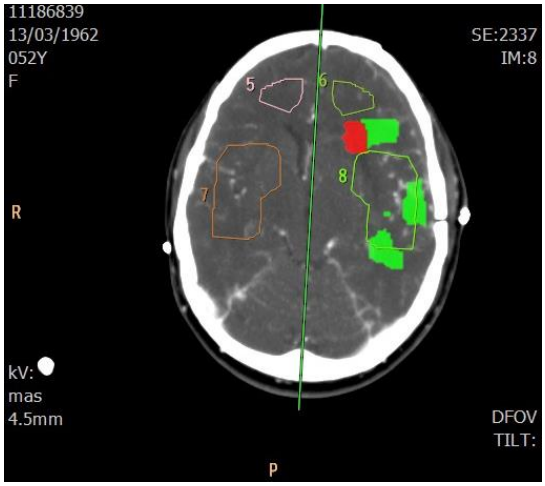


Fig. 13 a and b show a sample of a transcranial Doppler TCD bed-side examination of 1 patient included in the study





N° ROI	CBV (ml/100 g)	CBF (ml/100 g/min)	MTT	TTP
5	2,61	27,42	5,71	19,22
6	3,21	33,11	5,82	19,14
7	3,70	48,16	4,61	18,20
8	2,77	23,71	7,01	20,09

Fig 14.a and b shows severe MCA VS in a patient included in the study. The perfusion CT scan of patient with documented VS is showed. Regions of Interest (ROI) and values of MTT, CBV, CBF and TTP. This patient developed severe VS on day 7. This patient was the second included into the study

3. Sampling and quantification of TGF-β

We designed the study to detect early biochemical signs on EBI eventually associated to DINDs, therefore we restricted the temporal window to the first 7 days, before the maximum peak of VS risk.

Blood and CSF samples were collected at three time points: time zero (To) within 24 hours from bleeding, Time 1 (1) on day 3 and time 2 (T2) that was on day 7 and , when possible, matched with data from perfusion studies (Perfusion CT) as shown in Figure 15.

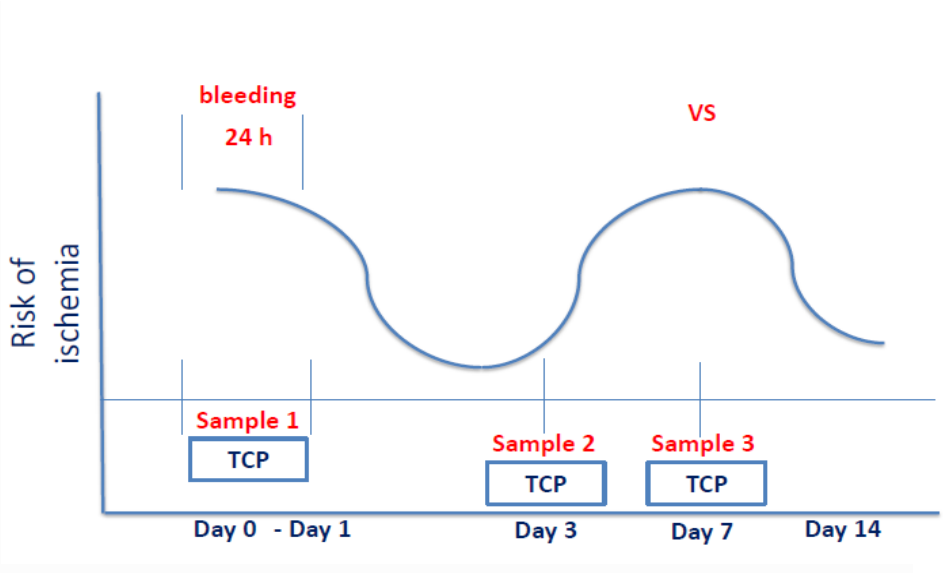


Figure 15. This scheme shows the two peck of ischemia risk, namely EBI and DINDS. Samples were drawn on day 1, 3 and 7 from arterial line and from the EVD proximal to patient's head statim

Blood samples were taken in a standardised method from an arterial line and stored in vials with Ethylenediaminetetraacetic Acid – EDTA, then, immediately centrifuged at 2000 rpm at 4 degrees for 20 minutes and then surnatants were cryostored at -80°C. CSF samples were drawn simultaneously from the EDV and equally processed.

TGFβ1 was quantified with an antibody sandwich enzyme-linked immunosorbent assay (ELISA), using antibodies that recognize only the biologically active molecule. The ELISA KIT used for quantification of TGF-β was the TGFβ1 Emax® ImmunoAssay System (Promega, Southampton, U.K.). The TGFβ1 Emax® ImmunoAssay System is designed for the sensitive and specific detection of biologically active TGFβ1 in an antibody sandwich format. In this format, flat-bottom 96-well plates are coated with TGF-β Coat mAb, which binds soluble TGFβ1, the captured TGFβ1 is bound by a second specific polyclonal antibody (pAb) and after washing, the amount of specifically bound pAb is detected using a species-specific antibody conjugated to horseradish peroxidase (TGFβ HRP Conjugate) as a tertiary reactant. The unbound conjugate is removed by washing, and following an incubation with a chromogenic substrate, the colour change is measured.

All non-manipulated CSF and blood samples provided values of biologically active TGFβ1 and acidified samples, in which latent TGFβ1 was activated, gave values of total TGFβ1 (1.5 µL 1N HCl per 50 µL CSF, incubated at room temperature for 15 minutes,

followed by neutralization with 1 µL 1N NaOH per 50 µL CSF). Figure 17 shows the check of successful acidification and neutralization results. In line with previous studies existing in literature on TGFβ1 quantification with this kit, we assayed different dilutions of CSF in Promega TGFβ1 sample buffer were to obtain an optical density for the range of 0.2 to 0.4 for which the standard curve was most accurate.

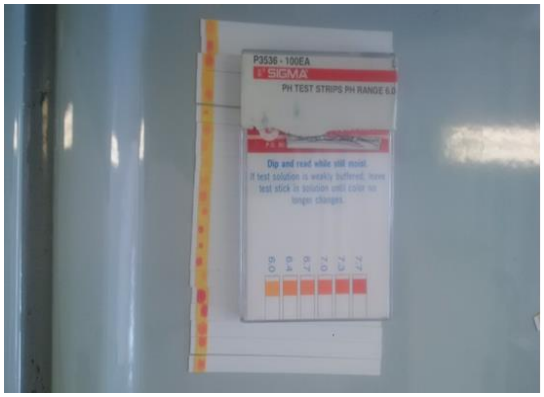
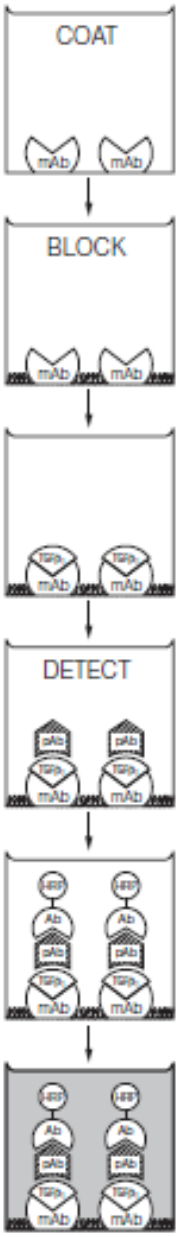


Fig. 16 and 17. Manual pH measure results.

The schematic procedure of protocol picture 18 showed below:



Coat well with TGFβ Coat mAb.

Prepare coating antibody. For each 96-well plate, mix 10µl TGFβ Coat mAb + 10ml carbonate coating buffer. Add 100µl/well. Incubate overnight at 4°C **without** shaking.

Block nonspecific binding with TGFβ Block 1X Buffer.

Prepare TGFβ Block 1X Buffer. For each plate, mix 22.4ml deionized water + 5.6ml TGFβ Block 5X Buffer. Add 270µl/well. Incubate **without** shaking for 35 minutes at 37°C; wash once. Prepare TGFβ Sample 1X Buffer. For each plate, mix 20.7ml deionized water + 2.3ml TGFβ Sample 10X Buffer.

Incubate immobilized TGFβ Coat mAb with $TGF\beta_1$ Standard or test samples.

Prepare standard curve. Dilute $TGF\beta_1$ Standard 1:1,000 with TGFβ Sample 1X Buffer. Add 200µl to row A, columns 11 and 12 of a 96-well plate. Perform six 1:2 serial dilutions. Prepare similar sample dilutions. Each well should contain 100µl. Incubate with shaking for 1.5 hours at room temperature; wash five times.

Incubate with Anti-$TGF\beta_1$ pAb.

Prepare second antibody. For each 96-well plate, mix 10µl Anti-$TGF\beta_1$ pAb + 10ml TGFβ Sample 1X Buffer. Add 100µl/well. Incubate with shaking for 2 hours at room temperature; wash five times.

Incubate with TGFβ HRP Conjugate.

Prepare TGFβ HRP Conjugate. For each 96-well plate, mix 100µl of TGFβ HRP Conjugate + 9.9ml TGFβ Sample 1X Buffer. Add 100µl/well. Incubate with shaking for 2 hours at room temperature; wash 5 times. **Equilibrate TMB One Solution to room temperature during incubation.**

Add TMB One Solution followed by stop solution.

For each plate, add 100µl/well room temperature TMB One Solution. Incubate **without** shaking for 15 minutes at room temperature.

Stop reaction with 100µl/well 1N hydrochloric acid. Measure A_{450}. **Read plate within 30 minutes.**

The TGF-β analysis was performed in a two-step protocol. Firstly, we sought to standardise a method of analysis. Therefore, we processed a random series of samples (blood and CSF). Any sample was diluted by a factor of 50-100 and 150 and the 300 and 500 (the latter for CSF only) and duplicated on plates.

According to the results and in line with previous studies described in literature, we applied two dilutions for blood and CSF by a factor of 300 and 500 folds.

Then the immunoassay TGFβ ELISA kit protocol was applied on nineteen patients included into the study. An expert scientist (dr. SB) blind to demographic, clinical data and outcome results, accomplished and supervised the analysis. Standard curves were generated to check if the experiment was correctly performed.

Statistical analysis

Categorical variables were described as frequencies and percentages, and continuous variables by mean values and standard deviations, or using median values . Comparison of categorical variables across groups was performed through the Fisher's exact test. Continuous variables were compared using the *t*-test, or the non-parametric Brown-Mood's test for medians. A P-value of <0.05 was considered significant. All statistical analyses were performed with Statview software, version 5.0.

Initially, we calculated the expression in CSF and Blood at the three time points in the whole population. Then we spitted results in single subjects.

Trends within single patients were individuated. The levels of TGF-β were compared between patients accounting for the initial haemorrhage severity scores (HH, Fisher and WFNS) and presence of warning bleeding.

We matched the TGF-β expression with the occurrence of VS and seizures requiring treatment with antiepileptic drugs (and if yes, we labelled any adjunctive antiepileptic drug (AED) as "Y". That means that patients with 3 AEDs were labelled as "YYY". Probability values of $P < 0.05$ were considered statistically significant. Comparisons between groups at To t1 and T2 were analysed using the Kruskal–Wallis test for the non-parametric analysis of the medians if indicated.

At baseline and over time, inflammatory activation due to triggers other than SAH was monitored with clinical and laboratory data. White blood cells count and indexes of systemic inflammation mainly triggered from infections were recorded.

The GOS and the variation of ischemic appearance of CT scan were analysed in single patients and correlated to the TGF beta trends.

4. Outcome and Quantification of ischemic burden on CT scan with the ASPECTS

We adopted two measure of outcome: the Glasgow outcome scale – GOS and the radiological appearance at CT scan at the time of admission, at any time point were possible and on discharge with the ASPECTS score.

The table below describes the GOS

1. Death	Severe injury or death without recovery of consciousness
2. Persistent vegetative state	Severe damage with prolonged state of unresponsiveness and a lack of higher mental functions
3. Severe disability	Severe injury with permanent need for help with daily living
4. Moderate disability	No need for assistance in everyday life, employment is possible but may require special equipment.
5. Low disability	Light damage with minor neurological and psychological deficits.

Modified from Jennett, B; Bond, M (Mar 1, 1975). "Assessment of outcome after severe brain damage". Lancet. 1 (7905): 480–484

In order to measure in a standardized way the CT scan appearance at the time of NICU discharge, we decided to use the Alberta Stroke Program Early Computed Tomography Score ASPECT score. This score is relatively novel, not widely used and we aimed to a standard methodology to compare the residual ischemic burden following SAH in a reproducible way between two observers. Because many patients had follow up up to one year following the admission to our NICU, we included all the available CT scan for any single subject included into the study.

A Neuroradiologist (GDA), blind to results of the study independently reviewed the non-contrast CT scans at the time of admission and on discharge to quantify the ischemic burden in single patients. ASPECTS score is a 10-item score assessing brain parenchyma hypodensity in predefined anterior circulation regions on brain non-contrast CT as a marker of early ischemic changes. Use of the score was part of the imaging inclusion criteria in several large randomized clinical trials on endovascular clot retrieval in ischemic stroke. The ASPECTS score is widely used to identify acute ischemic stroke patients with established infarction who may not benefit or may be harmed by reperfusion therapy. Historically ASPECTS is used to drive neurologists in the decision making process towards intravenous thrombolysis in patients with ischemic stroke.

To our knowledge, there are no current studies on the values of TGF-β trends in detecting EBI and DINDs matched with this radiological scale. It is important to stress that the diagnosis and

monitoring of treatment of VS was obviously made with perfusion CT while the ASPECTS was intended to be only a methodological tool to review CT scans.

We decided to apply this measure of outcome because the inter- and intra-observer reliability of ASPECTS has been demonstrated to be good to excellent (κ = .71–.89) and consistently superior to the 1/3 rule between observer pairs from the same specialty. ASPECTS is used in practice, it can be used on CT scans obtained on all commonly used axial baselines, and emphasize that clinicians from different specialties and with varying levels of experience in assessing acute ischemic CT changes can agree on an acceptable level.

The method essentially consists in dividing the MCA artery in 10 zones (fig 19).

One point is subtracted per infarcted zone (hypodensity). If the score is 7 the ischemia is less than 1/3 of parenchyma.

The ASPECTS value is calculated from 2 standard CT scan cuts:

- At the level of the thalamus and basal ganglia
- Just rostral to the basal ganglia

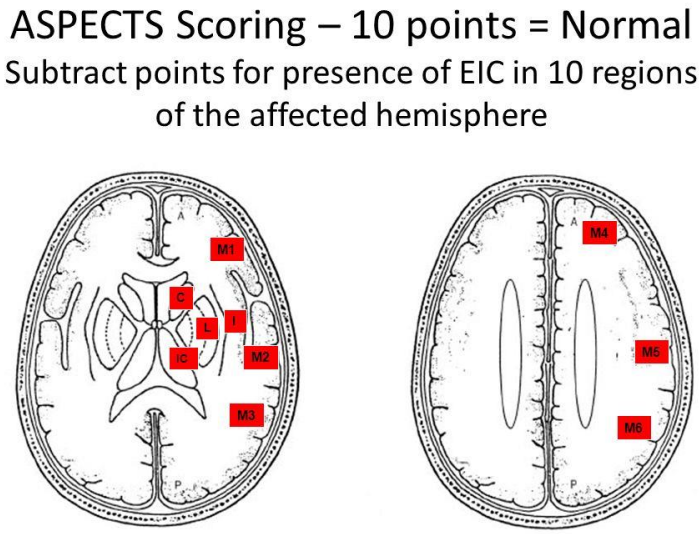


Fig 19. ASPECTS scoring

Parenchymal hypo-attenuation was defined as a region of abnormally decreased attenuation of brain structures relative to attenuation of other parts of the same structures or of the contralateral hemisphere. Focal brain swelling or mass effect was defined as any focal narrowing of the CSF space due to compression by adjacent structures, such as effacement of cortical sulci or ventricular compression.

A normal CT scan received an ASPECTS of 10 points. A score of zero indicated diffuse ischemic involvement throughout the MCA territory. In this study, the ASPECTS was arbitrarily set at 10 on the initial CT scan unless severe diffuse ischemia was present in part of the brain far from the bleeding.

Chapter IV

Results

Chapter IV Results

1. Population characteristics

2. TGF-β levels trend over time and across patients

3. Association of TGF-β to drugs, infections, antipyretics drug and treatment

4. The ischemic burden quantified with the ASPECTS score

1. Population characteristics

Results have been restricted to a small number of patients derived from a cohort of 52. Ten patients dropped out from the study because they died before day 7. That means that we might have lost the more severe. Twelve patients had EVD inserted with issues on sampling (i.e. obstruction) or removed before the completion of the study. Finally, in 11 subjects for whom consent to participate that study was obtained technical issues impeded a correct sampling.

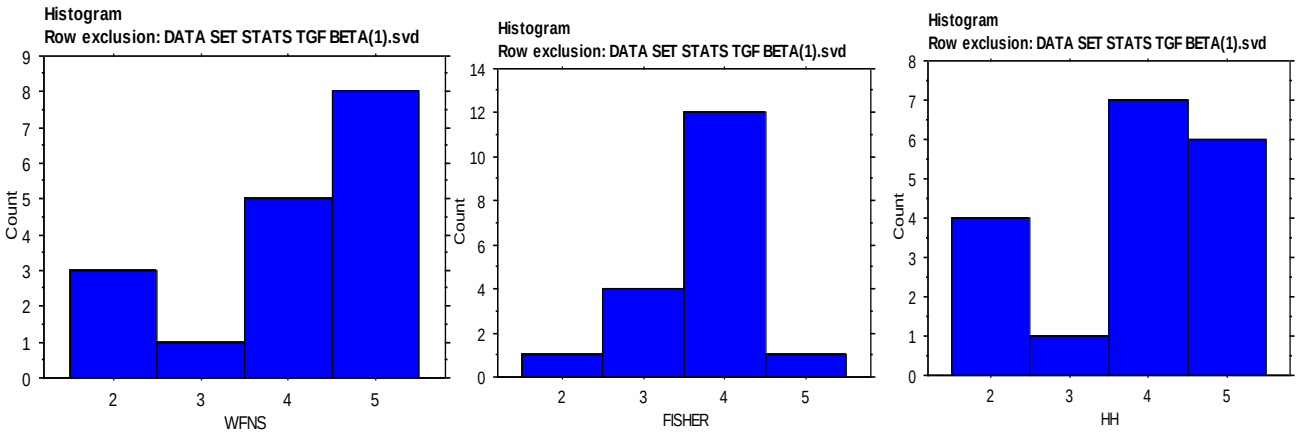
Nineteen patients were studied, 14 females, 5 males. Media age 56.5 , median 55.

Median GCS at the time of bleeding was 7, 13 patients (68%) were intubated at the scene. Median length of stay (LOS) in ICU was 19 days. Three patients had a LOS in NICU of 8, 9 and 13 days respectively because they died on the day after (patient 9, 11 and 13). Sedation, infusion of diclofenac sodium, paracetamol, antibiotics and thrombolytic drugs were detailed. Nine (47%) patients experienced the warning bleeding, 8 (42%) patients underwent tracheostomy. Eleven (58%) patients had seizures and 14 (74%) had VS, of whom 9 with seizures.

Demographics, treatment, complications and GOS are shown in the tables 1.a and b and table 2 below.

N	Gend	AGE	TI	Fisher	WFNS	aSAH	treatment	DC	HH
1	F	48	N	4	1	PICA DX	COILING	N	2
2	F	52	Y	4	5	MCA SIN	CLIP	Y	4
3	F	55	Y	4	5	PICA SX	COILING	Y	5
4	F	66	Y	4	4	ACO A	COILING	N	4
5	M	46	N	4	4	ACOA	COILING	N	3
6	F	71	Y	4	5	PICA DX	COILING	N	5
7	M	61	Y	4	5	ACO A	COILING	N	5
8	M	44	Y	4	5	ICA	COILING	N	5
9	F	28	Y	3	5	ICA	COILING	Y	4
10	F	68	N	4	2	ACA -MCA	COILING	N	2
11	F	42	Y	4	4	MCA SIN	CLIP	Y	4
12	F	60	Y	4	4	MCA DX	CLIP	Y	4
13	F	48	Y	4	4	MCA SIN	CLIP	Y	5
14	M	61	Y	4	4	ACA	COILING	N	5
15	F	73	N	2	4	ICA	COILING	N	3
16	F	53	Y	4	5	MCA DX	CLIP	Y	5
17	M	67	N	4	2	Bas	COILING	N	2
18	F	85	Y	4	4	ACA	COILING	N	4
19	F	46	N	2	1	MCA	COILING	N	2

Tab 1.a Gend is gender. TI-tracheal intubation at the scene, DC – decompressive craniectomy. Details are split in the table 1.b



Tab 1.b The picture shows distribution of patients within the gravity score

warning bleeding	Tracheostomy	EEG	VS	days in ICU	GOS
Y	N	N	N	12	5
N	Y	YY	Y	30	4
Y	Y	YYY	Y	28	2
Y	Y	N	Y	30	4
Y	N	N	N	13	5
Y	Y	YYY	N	30	2
N	N	YY	N	16	5
N	N	Y	N	15	3
N	N	N	Y	8	1
Y	Y	Y	Y	28	4
Y	N	Y	Y	9	1
Y	N	Y	Y	21	4
Y	N	YY	Y	15	1
N	Y	Y	Y	15	3
N	N	N	N	14	5
N	Y	YY	N	20	2
N	N	N	N	33	4
N	Y	N	Y	20	3
N	N	N	Y	21	5

Tab 2

We recorded the white blood cells count daily and, were indicated on medical basis, indexes of infection and inflammation were also recorded (C-reactive protein and Procalcitonin) (tab 3.)

Only 5 patients developed infections within 7 days and were put on antibiotics. One patient died because of septic shock at day nine.

Fever in single patients was actively counteract with ultra-early Diclofenac sodium continuous 75 mg/day infusion as soon as core temperature raised above 37.8°. Mean and median temperatures on the day of the sample and within the seven days of observation was measured. No patients experienced significative burden of fever (according with the established cut off of 38.2°C in literature) during the seven days period of observation.

WBC To	WBC T1	WBC T2	PCT To	PCT T1	PCT T2	PCR To	PCR T1	PCR T2
12.490	9880	8000	0.04	-	0.05	1.29	2.5	N
21000	24000	14000	-	0.16	0.12	-	21	9
7800	9800	13000	-	-	0.17	-	-	-
7000	12000	7000	0,06	-	0,07	-	-	-
12000	10000	8600	-	-	-	4	-	3
18000	22000	15000	0.09	0.42	0.12	4	3	-
13000	15000	10000	-	-	0.12	0.16	10	
11600	8910	9000	0.5	-	0.44	1.88	19.12	43
16000	20000	1200	-	-	-	8.3	20	-
15000	8900	8000	-	0.7	-	6	28	-
10000	13000	9000	-	-	0.04	4.2	4	-
6000	8000	11000	0.1	-	0.12	-	-	-
13000	14000	8000	-	-	0,6	0,76	9	1,74
8000	11200	5600	-	0.1	0.07	-	10	7.5
11200	15000	7000	-	0.35	-	0	0	-
10900	14000	13000	-	-	0.14	2	4	-
20000	18000	20000	-	-	-	-	-	-
12000	10000	11000	-	0.2	-	-	5	-
12000	12.500	8000	-	0.35	-	3	-	-

Table 3. Blood test on day 0, on day 3 and seven.

SEDATION To	SEDATION T1	SEDATION T2	DCF To	DCF T1	DCF T2
P+F	N	N	Y	Y	Y
P+F	P+F	F	N	N	Y
P+F	KETA + MDZ	KETA + MDZ	N	Y	Y
P+F	MDZ + F	KETA + MDZ	N	Y	Y
P+F	N	F	N	Y	Y
P+F	P+F	N	N	Y	Y
P+F	P	N	N	Y	Y
P+F	P+F+BZD	P+F+BZD	N	Y	Y
P+F	P+F	MDZ + F	Y	Y	Y
P+ F	N	N	Y	Y	Y
P + F	MD E KETA	MDZ K	N	Y	Y
P + F	P + F	P + F	N	Y	Y
P+F	MDZ + F	MDZ + F	N	Y	Y
P+F	P+F	P+F	N	N	Y
N	N	N	N	N	N
P+F	N	N	N	Y	Y
N	N	N	N	Y	Y
P	N	N	N	N	Y
N	N	N	N	N	Y

Tab 4. Sedation: P is propofol ; F is fentanyl; Keta is Ketamine and MDZ is midazolam. DCF is diclofenac

Continuous and quantitative EEG monitoring was used if patients suffered from seizures documented on clinical ground or on continuous EEG when comatose, any drug was recorded as Y (yes). Three patients had 3 antiepileptic drugs and sedation because on no convulsive status epilepticus (YYY). Patients follow up was monitored from the time to ICU discharge until September 2017.

2. TGF-β levels trend over time and across patients

The primary outcome of interest was to disclose if the expression in TGF-β in CSF and plasma showed a trend over the seven days period of sampling. Continuous data are presented as raw data (mean ± SD). We also tested the presence of levels of the biomarker between SAH severity groups and healthy controls (internal control data not shown). A total of 114 samples of CSF and blood were analysed.

Firstly, we calculated the expression in CSF and Blood at the three time points in the whole population. Then we spitted results in single subjects.

A standard curve for TGFβ1 (0 to 2,500 pg/mL) was generated using recombinant rat TGFβ1 and the assay was performed according to the manufacturer's instructions. Absorbance was

read at 450 nm using a Synergy 2 microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA). Absorbance was also measured at 550 nm to correct for optical imperfections in the assay plate.

The coloration shows that the experiment was correctly performed according to the manufacturer instructions, Fig 19.

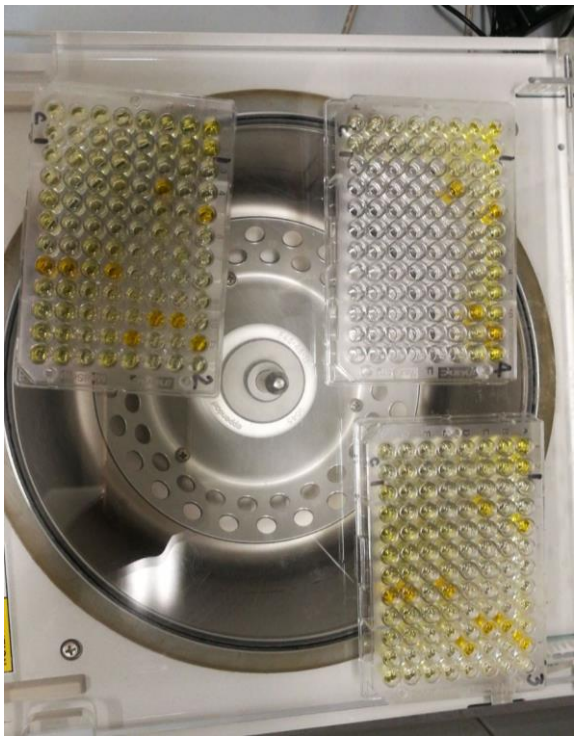


Fig 19. a

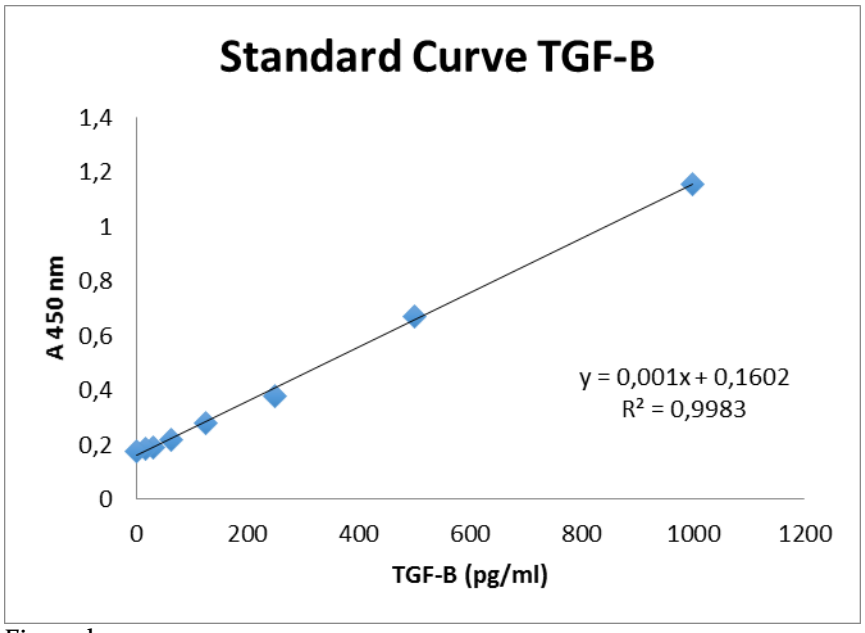


Fig 19. b

Fig 19 a and b. The latest step of the experiment was to stop the reaction and consisted in the acidification by adding 100µl of 1N hydrochloric acid to the wells . Blue changes to yellow upon acidification and the absorbance is recorded at 450nm on a plate reader within 30 minutes of stopping the reaction.

The concentrations of TGF-β was expressed in pg/mL. Mean CSF levels of total TGF-β were 18895, 28005 (sd 6733,531033) at time zero (To), 6179,83907 (sd 2172,089535) at T1 and 15043,0186 (sd 7373,43863) at T2. Mean concentrations in plasma were 58084,43 (sd 7613,381) pg/mL at To, 47973,79 (sd 6659,051) pg/mL at T1 and 57877,81 (sd 10595,13) pg/mL at T2.

All raw data in pg/mL are reported, some patients had values of TGF-β in CSF below the level of kit sensibility (32 pg/mL):

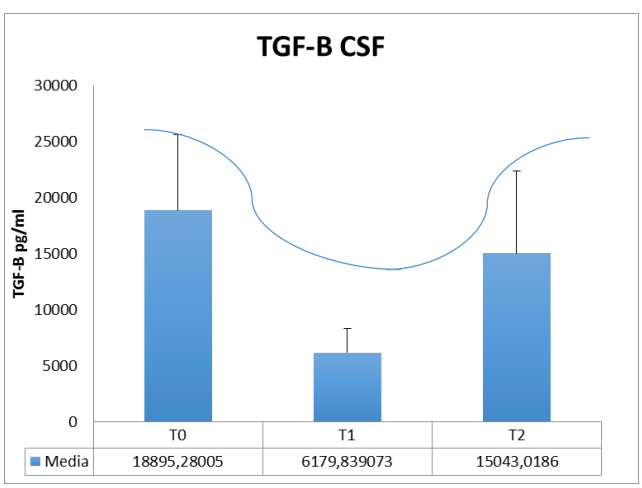
CSF					Plasma		
T0	***T1***	***T2***			***T0***	***T1***	***T2***
8800,000	6859,699	52699,996			28965,001	28260,004	52604,999
76600,000	7319,249	12024,999			72015,001	41234,996	32220,000
12550,000	1325,000	950,001			75900,002	12465,001	66615,003
16324,999	3200,002	7350,001			13725,000	27149,999	27554,999
6625,002	1572,501	5749,997			73934,998	16395,000	182490,000
9699,999	607,500	98974,994			48704,999	67604,998	71579,999
7700,002	33025,002	600,001			21525,001	65684,999	52995,000
3545,455	1299,998	2325,000			108150,001	106679,999	32189,999
65454,547	2545,455	2272,730			111389,994	37335,003	10605,000
1727,273	2684,091	4545,455			56970,000	60765,002	5430,002
1590,910	3840,911	2654,544			83610,002	7679,999	21165,000
28295,454	8054,546	13840,908			71481,820	79590,001	64418,182
6725,000	2477,272	1727,273			19486,363	62209,094	71195,453
	15886,363	4886,363			35072,726	57409,090	27327,273
	1999,998				72629,999	91213,633	112090,907
					35790,001	14986,364	102374,999
						50745,003	51066,000
						36119,998	

Tab 5. Raw data of the TGF-β concentration across population, values in pg/mL.

These results are in line with other described in literature. Differences in TGFβ1 mean levels between time intervals was established by t-test. Differences in the mean values of TGF-β in

CSF and plasma at To, T1 and T2 were statistically significant ($p<0.05$).

The bar chart shows the cumulative trend in the population.



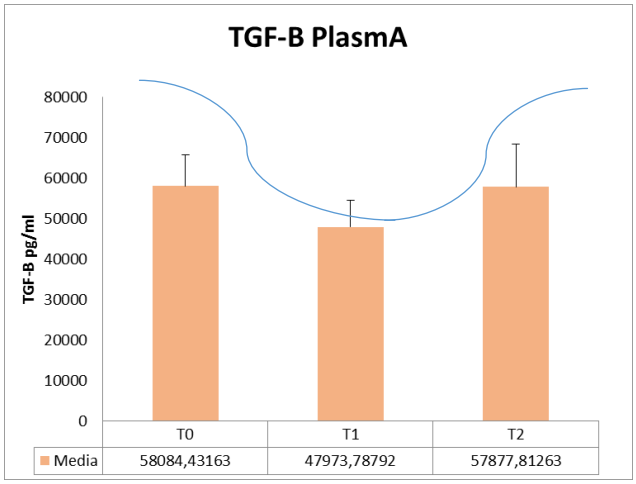


Fig 20. Main result of the study. TGF trend over seven days across the population

At that point, trends within single patients were individuated.

The levels of TGF-β was compared between patients accounting for the initial haemorrhage severity scores (HH, Fisher and WFNS) and presence of warning bleeding.

There was no difference in TGF-β expression in CSF at To among patients who experienced warning bleeding.

Severity at the exordium was defined as low GCS (<8), high Fisher and the documented seizures at the scene.

Comatose patients had higher levels at To in CSF and plasma as shown in picture below with cumulative range above 2000 pg/ml ($p<0.05$)

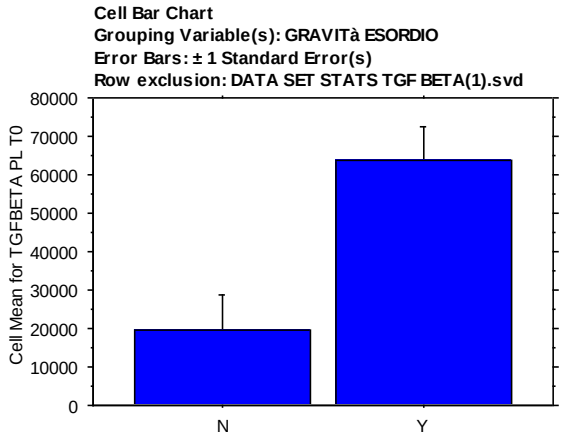


Fig 21 Severity at the exordium

However, these higher levels of TGF-β in plasma at To did not correlate with outcome (see later).

Within the population we have observed two trends in TGF-β expression. Therefore, patients were dichotomized by whether there was an increase or decrease of TGF-β in T1 compared to T2. Univariate analysis was performed to detect differences in clinical variables between the groups by student t and Fisher exact test.

In 14 patients, VS occurred (> 75%) in 9 (64%) of those with a TGF-β trend characterized by a drop in T1 (as shown in the general trend of the population) with an increase toward T2 in CSF. This trend is defined as "trend 1" and was the general trend observed in the population and in the majority of patients with VS.

The picture below shows the trend over the population of TGF-β values in patients with and without VS

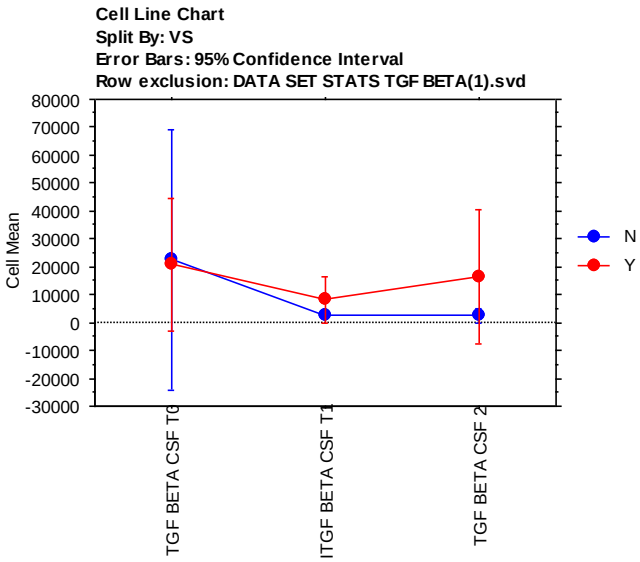
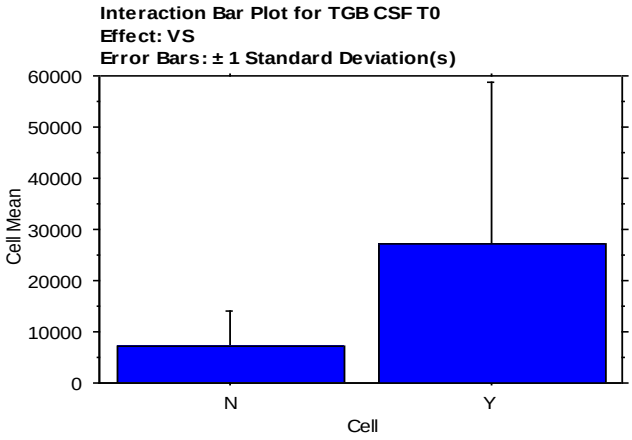
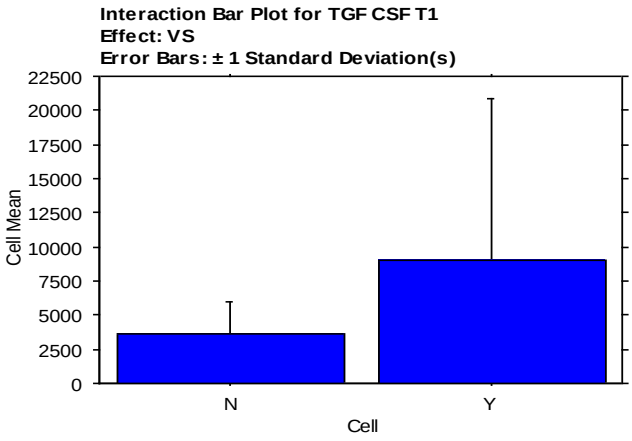


Fig. 22. General trend of TGF-β in CSF in patients with VS (Y) and not (N)

The mean values of all CSF concentration of TGF-β in patients that developed VS was statistically different with $p<0.05$. Mean VS time of occurrence was between day 7 and 11 (fig 21).


Interaction Bar Plot for TGB CSF T0
Effect: VS
Error Bars: ± 1 Standard Deviation(s)
60000
50000
40000
30000
20000
10000
0
Cell Mean
N
Y
Cell


Interaction Bar Plot for TGF CSF T1
Effect: VS
Error Bars: ± 1 Standard Deviation(s)
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
Cell Mean
N
Y
Cell

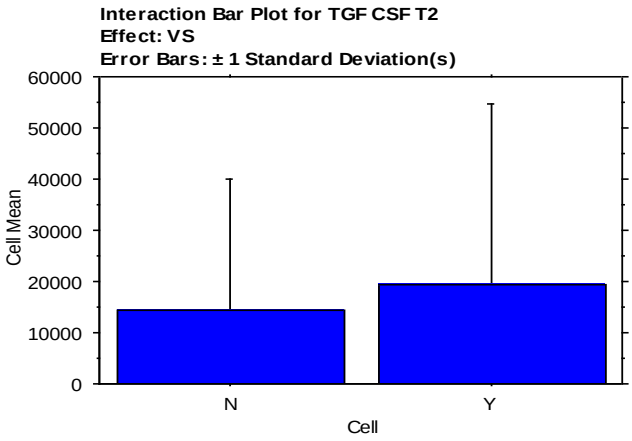

Interaction Bar Plot for TGF CSF T2
Effect: VS
Error Bars: ± 1 Standard Deviation(s)
60000
50000
40000
30000
20000
10000
0
Cell Mean
N
Y
Cell

Fig. 23 a,b and c show trend in CSF into the 3 time points in patients with and without VS. The bar chart in detail shows that at any time point in 64% of patients who developed VS the value was higher.

There were differences in TGF-β expression in T2 in blood and CSF between patients who developed VS and whom that did not but p was close to 0.05 not below (fig 22).

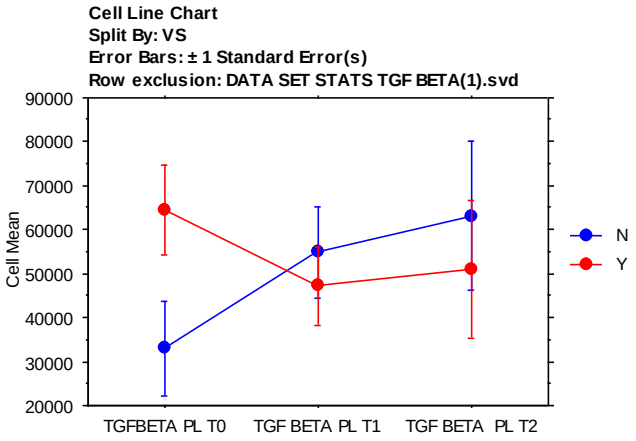


Figure 24. TGF in plasma in patients with VS and without VS.

Successively, we divided population of those who developed VS and we found that mean values of TGF-β in CSF were higher within Fisher groups as shown below. Values above 12000 pg/ml were associated to VS at T2 in CSF (T-test for means and equality of variance for median values $p<0.05$)

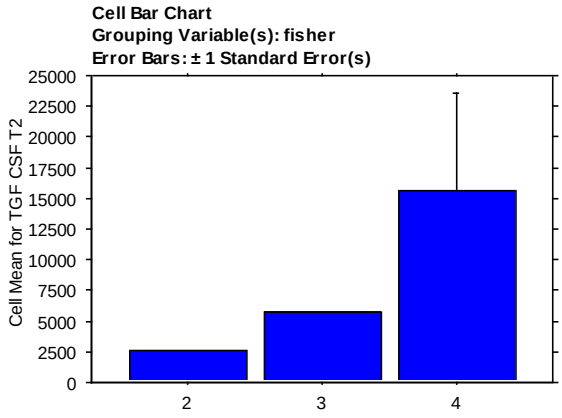


Fig 25 Fisher and VS at T2

In 5 patients with VS without the TGF-β trend 1, we had: 2 patients without TGF-β results (the values were not detected by the assay for any reason) and 3 had a second pattern named "trend 2".

The second trend that we noticed in 5 patients was measured with a peak in T1 and a then a drop in T2. Of those patients: 2 had massive VS and cerebral ischemia (described in details below), two had severe EVD obstruction but a good recovery with no DINDs and 1 patient had low and equal levels at the 3 time points. This subject died within NICU on day 14.

Results of TGF-β concentrations in CSF have been restricted to 15 patients

Patients	Trend 1 T1<t2	Pattern 2 T1> T2	Variable results
VS 14 (75%)	9 (64%)	2 –brain death	1 – brain death 2- no TGF
No VS 5 (25%)	1	2 –HC	2 –no TGF

Pattern of TGF-β in CSF. HC- hydrocephalous.

Of those patients who died in NICU (3) the second trend was observed with significant difference of TGF-β values between T1 and T2 ($P< 0.05$) in 2 subjects.

Instead, one patient died in ICU on day 14 and had severe sneaky ischemia from day 4. The first CT scan appearance with ASPECTS of 9 dropped to 7 on day 5 when VS occurred. Despite this CT scan appearance, a continuous nimodipine infusion was administered. Massive cerebral oedema occurred on day 8 and patients went on brain death on day 13. This patient trend of TGF-β in CSF is showed in figure 23.

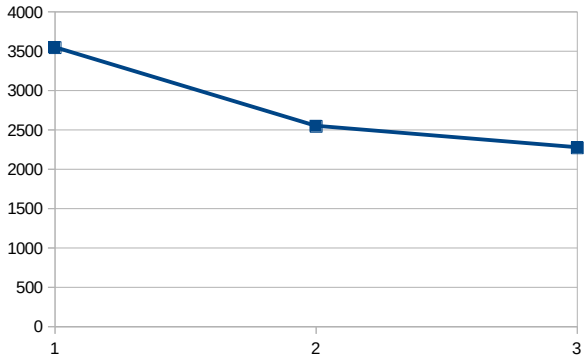


Fig 26. This patient had very low levels of TGF-β and there was no difference between T1 and T2. Severe ischemia was documented on CT scan from T1.

In 2 patients who died because of massive ischemia and with the trend 1, TGF-β in CSF peaked at T1 at concentrations above 6000 pg/ml and dropped to very low levels on day 7. Both patients had the trend showed in figure 2z a and b, and died within day 8 and 10 post SAH (fig 27 a and b shows the CT appearance on To-1 and 2).

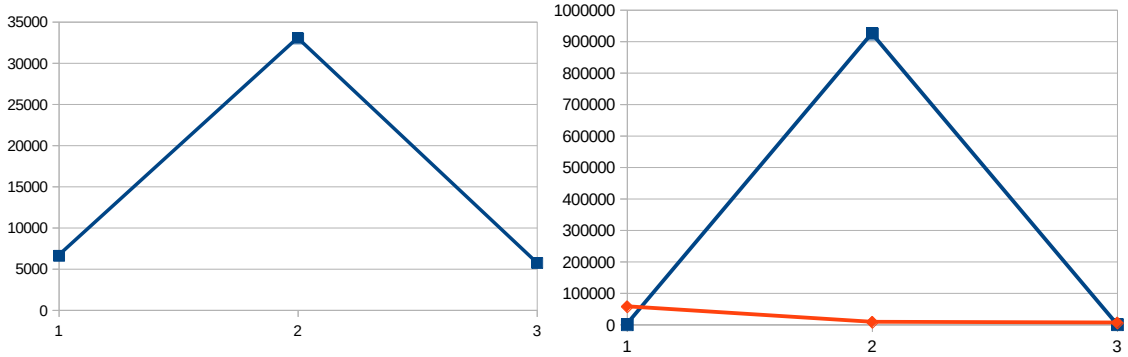


Fig 27.a

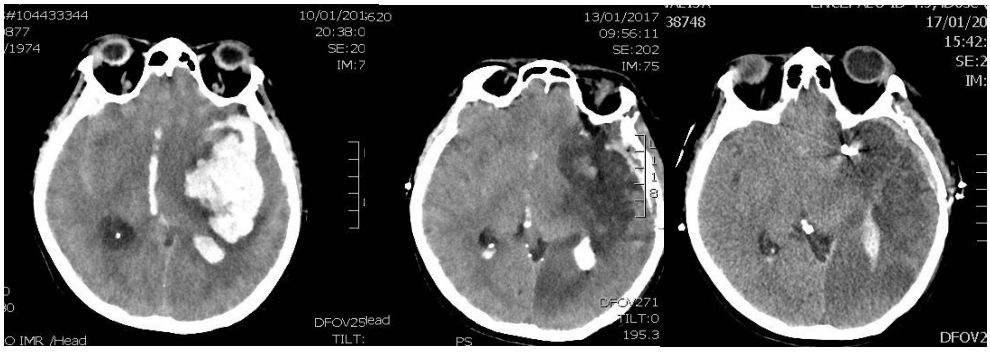


Fig 27a and 27b: ASPECTS was 9 at the time of bleeding, then dropped to 7 at T1 and then went to 1-0 at the time of brain death. ASPECTS was 7 on the left side at T1 and zero at T2. Non benefit was reached by VS treatment with the rescue therapy with intra-arterial nimodipine

In the remaining 2 patients with a peak of TGF-β in T1 (pattern 2) and with the outcome good, levels of TGF-β in CSF increased in concomitance of EVD obstruction (acute HC) and declined over time when obstruction was sorted.

Previous studies available in literature were able to demonstrate that patients that went on to develop chronic communicating hydrocephalus because of haemorrhage have significantly higher levels of total TGFβ1 as compared with those patients with CH for other reasons. In our population, five patients developed CH, CSF levels did not differ compared to other subjects. The other studies associated the CH to sustained levels of TGF-β in CSF until day 19 and not only in the acute phase. Therefore, this comparison cannot be made in this study.

Seizures

TGF-β expression in CSF varied among patients who had seizures. Generally, TGF-β expression in patients with positive EEG was higher as compared to those who had no positive EEG. This difference was statistically significant (p<0.05). However, this is not surprising as the majority of patients had contextually VS.

We then split the population of patients who had severe seizure and underwent periods of burst suppression because of continuous non-convulsive activity on continuous EEG. These patients had lower cumulative TGF-β expression in CSF as compared to patients that were less severe.

We found this general trend of TGF-β expression within patients with or without positive EEG as shown in picture28

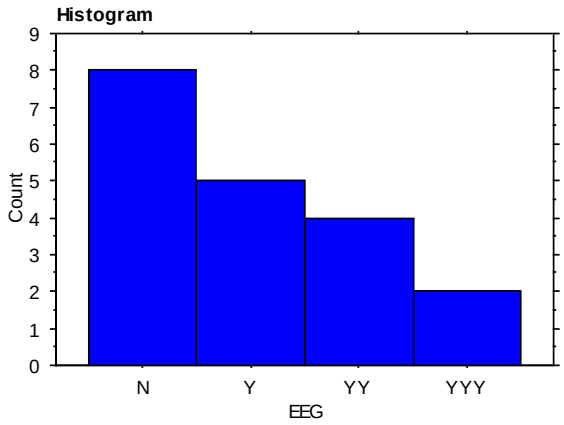


Fig. 28. "Y" means one AED, "YY" 2 AEDS, "YYY AEDs", "N" no AED.

3. Association of TGF-β to drugs, infections, antipyretics drug and treatment.

Influences of drugs in NICU on TGF-β expression: Opiates, Propofol, Midazolam and sedation had no effect on TGF-β expression.

Influence of aneurysmal treatment on TGF-β expression showed a trend towards increase in plasma at T1 but not in CSF in patients who underwent craniotomy (clipping or intracerebral haematoma removal). Diclofenac continuous infusion was recorded. No patients were allowed to sustain fever for more

than 2 h. Only 2 patients had DCF infusion on day 1, fourteen patients had DCF infusion on day 3 and 17 on day 7.

At baseline and over time, inflammatory activation due to triggers other than SAH was monitored with clinical and laboratory data. White blood cells (WBC) count and indexes of systemic inflammation mainly triggered from infections were recorded. Comparisons between groups at To, t1 and T2 were analysed using T-test. No correlation was found in TGF-β increase and WBC count, expression of CRP and Procalcitonin neither with antibiotic therapy starting.

We dichotomized the values of TGF-β across patients who had WBC above the range of normality of our laboratory (12000/mm3). At To-T1 and T2 there was not any statistically significance difference in TGF-β values in plasma and CSF of patients with increased WBC (> 12000). Therefore, there was not any influence of WBC count on TGF-β expression. We separately matched the values of TGF-β in CSF and plasma at the time points were CRP and PCT were increased. No influence of systemic activation was found.

In respect of the outcome we failed in identify a single cut off of TGF-β that could be predictive of dead or unfavourable outcome. However, the 2 patients that suffered from severe ischemia from day 3 (and then died in NICU) were the only who experienced a TGF-β increase with a step above 6000 pg/ml.

In light of the previous reports, we tried to generate a cut off to predict VS. We set the value of TGF-β in CSF at 3000 pg/mL, an arbitrary level in between the median values of TGF-β expression in the entire population at To. We found that in those patients who had VS the TGF-β at To was above 3000 in 65% of patients.

The presence of values at > 3000 pg/mL of TGF-β expression showed sensibility of 81% with a confidence interval of (likelihood ratio) 90% and low specificity to 60% (Bayes Theorem) in patients who developed VS but the sample was too small and P was above 0.05

4. The ischemic burden quantified with the ASPECTS score

The ischemic burden across single patients was calculated as a measure of outcome in single patients with the ASPECTS score.

Parenchymal hypo-attenuation was defined as a region of abnormally decreased attenuation of brain structures relative to attenuation of other parts of the same structures or of the contralateral hemisphere that was not detectable on previous scan. Focal brain swelling or mass effect was defined as any focal narrowing of the CSF space due to compression by adjacent

structures, such as effacement of cortical sulci or ventricular compression.

Given the fact that all CT scans were abnormal by definition in this population and that the methodology we tested is new, we generated a database in any single patient and we considered significant any new ischemia in territories (right and left) that were not interested (i.e. had a point of 1) by bleeding. Paradoxically, ASPECTS at time zero was defined as 10 in majority of patients. The 10 regions on the left and side were at the level of: caudate nucleus, insular ribbon, internal capsule, Lentiform nucleus, MCA territories divided into M1-2-3-4-5 and 6. We recorded the worst ASPECTS variation on the latest CT scan as an absolute value (a negative number). But then again, as we did for the TGF-β values, we split results within single subjects.

Two different observers analysed the CT Scan across the population from admission to discharge and 142 radiological examinations were reviewed.

As expected, the 3 patients who died in NICU experienced visible ischemia within day seven 7 with at least a score of -2 within day 4-5 (that means within the timing of T2).

Interestingly, in those patients who had TGF-β expression with the trend 1, the score ranged from 0 (no changes) to -2 (final ASPECTS score of minus two points from the first CT scan), regardless to the GOS and the presence of VS. In those with

Pattern 2, ASPECTS was lower and included values of <3 (tab 6.a and 6.b)

ASPECTS	VS	GOS	EEG
-1	N	5	N
-2	Y	4	YY
-2	Y	2	YYY
-2	Y	2	YYY
0	N	5	YY
-2	Y	3	Y
-1	Y	3	Y
-2	Y	3	YYY
0	Y	3	Y
-2	Y	2	YY
-2	Y	3	N
0	Y	5	N

Tab 6. a ASPECTS in TGF-β trend 1 of expression in CSF

ASPECTS	VS	GOS	EEG
-2	Y	2	YYY
-3	Y	3	Y
-3	Y	1	N
-4	Y	1	Y
-3	Y	1	YY
0	N	5	N#
0	N	4	N#

Tab 6. b, Some of the patients who showed TGF-β expression with the pattern 2 had scores from -3 points. The # means that these two patients had HC.

Chapter V

Discussion

Chapter V Discussion

1. The rationale underneath the choice of TGF-β is based on the anatomical district. The Microglia

2. The platform of previous studies

3. TGF-β expression and ischemic burden: vasospasm and documented changes on CT scan (the ASPECTS)

4. Conclusion, translational relevance and future perspectives

1. The rationale underneath the choice of TGF beta based on the anatomical district. The Microglia

The main brake to translation of preclinical research to clinical effectiveness is linked to the complex interaction of molecular pathways implicated in brain damage. Several randomized controlled trials on SAH aimed at counteract secondary brain damage, failed to confer a single neuroprotective strategy in this pathology.

This observation has been one of the main conclusion in reviewing the literature of the available biomarkers already investigated in remarkable studies.

We attempted to narrow our query and we reduced the focus at three single questions: what is one of the most prevalent and certain documented mechanism of DINDS following SAH? Is there any alternative pathway that has not been widely and fully investigated in literature? Are there any cells into the brain that contribute to secondary insult and do not belong to neuronal or macroglia architecture?

Inflammation, effectors of reparative strategies and wound healers and microglia fitted to our question.

Starting from the beginning, we considered the recent literature on microglia as described in chapter one.

Microglia are the resident macrophages of the central nervous system and are associated with the pathogenesis of many neurodegenerative and brain inflammatory diseases; the origin of adult microglia has been controversial since the 90's. In vivo lineage tracing studies established that adult microglia derive from primitive myeloid progenitors that arise before embryonic day 8.

Postnatal hematopoietic progenitors do not contribute to microglia homeostasis in the adult brain. In contrast to many macrophage populations, microglia develop in mice that lack colony stimulating factor-1. Then, microglia's embryonic primitive myeloid progenitors are distinct from the postnatal hematopoietic origin of other tissue macrophages and their numbers are maintained independently of circulating hematopoietic cells in the adult brain. Ginhoux et al. recently confirmed that microglia is an ontogenically distinct population in the mononuclear phagocyte system and that peripheral myeloid cells do not contribute to maintenance of adult microglia afterward (1). As said in chapter I, some Authors demonstrated the requirement of TGF-β for the in vitro development of microglia and that TGFβ1 deficient mice have no development of microglia in CNS. A unique microglial signature exists and is dependent on TGF-β signalling. It has been reported that in the experimental autoimmune encephalomyelitis (EAE) model, infiltrating monocytes do not contribute to the residual microglial pool and that microglia can be distinguished from monocytes

during neuroinflammation. In a number of disease, such as trauma, MG is stimulated and migrate to the area of injury, where they phagocytose debris. Other studies showed that TGF-β modulates microglial phenotype and promotes recovery after ICH. However, the neurotoxic versus protective properties of MG and the role of TGF-β are not fully elucidated across the broad spectrum of brain pathologies, mainly over the time course of different diseases.

2. The platform of previous studies

Using the platform of previous studies from Douglas et al. we examined the kinetics of TGF-β expression in sequential time points in CSF and blood of patients with SAH. The main differences with the mentioned paper are essentially three. Firstly, the aim of the study was to disclose the association of TGF-β with chronic communicating hydrocephalous. Second, in that study, CSF levels were compared between two cohorts of patients with chronic hydrocephalus caused by SAH or other reasons (namely seven controls). And third, the levels of CSF were averaged over time. That means that the temporal changes were recorded on day 1-5, 6-9, 10-14 and 15-19. Not every patient contributed to all samples (the same in our study).

In our paper we investigated the expression of TGF-β across the population and then in single patients at three precise time points (day 1-3 and 7) that are considered a very early phase in SAH, and not across the entire period of VS risk (until day 19-21). Blood and CSF were taken simultaneously. Our population was homogeneous and controls were not included. The main question of our study was to disclose a trend in TGF-β expression in SAH patients and to correlate it to the insurgence of late complications, mainly vasospasm and not communicating hydrocephalous.

The first result that matched the aim of the study was to track a temporal profile during early and delayed ischemic injury. CSF and plasma TGF-β levels had a variable expression at the time of the first sample, then drop in the majority of patients on day 3 and increased towards day seven.

In line with other studies in Literature, we started from the assumption that Platelets are certainly the source of TGF-β in the extravasated blood into the CSF at time 0. No patient experienced rebleeding. The sustained levels of TGF-β expression and the peak recorded on T2 in patients of our cohort suggests, in line with other studies, that the release is due to an endogenous response to irritation of the choroid plexus epithelium and leptomeningeal cells triggered by extravasated blood. The endogenous synthesis of TGF-β in our study was further confirmed by the fact that in those patients that

experienced VS the trend of the biomarker in T2 was the opposite in blood and CSF.

The second step in the analysis of TGF-β results was to split values across single patients and within categories of complications: vasospasm, seizures and chronic hydrocephalous.

Vasospasm

VS occurred in 75% patients and in 9 of those TGF-β trend was characterized by the drop in T1 with an increase toward T2 in CSF and we identified a trend (trend 1). No patient with trend 1 died within the NICU stay and up to 6 months of follow up. Across patents with TGF-β trend 1 in CSF the ASPECTS ranged from 0 to – 2. Among patients with trend 2, 3 patients with VS died within day 11 because of massive cerebral ischemia and TGF-β peaked dramatically on day 3 to levels above 6000 pg/mL, then massive VS took place with ongoing cerebral ischemia, required decompressive craniectomy for intractable intracranial hypertension also intra-arterial nimodipine as rescue therapy but brain death occurred. We found this result very interesting because these 2 patients were the unique in the population that experienced a step of TGF-β concentration between To and T1 > 6000 pg/mL. The third patient who died within day 13 had sustained levels of TGF-β and massive vasospasm with cerebral ischemia requiring decompressive craniectomy. We interpret

this trend as the result of malignant VS with severe status epilepticus that occurred from day 9.

In those with VS we had three results:

1. Absolute values in CSF were higher ($p< 0.05$).
2. At T2 the trend of TGF-β expression in CSF and blood of subjects with VS was the opposite as compared to blood, despite the difference in absolute numbers that expressed the TGF-β concentration were not statistically significant.
3. In five patients, TGF-β in plasma was markedly increased in T1 at the time were inflammation was documented by PCT or CRP but this increase was not associated to similar increase in CSF.

The effect of sedation was disclosed. Studies have addressed the correlations between propofol and TGFβ1 expression. We did not find differences in To and T1 levels of TGF-β of patients who were sedated at time zero and not sedated at time 1 (effect of discontinuing sedation). We dichotomized patients who had sedation and compared absolute levels of TGF-β across the three time points but we failed to disclose any statistically relevant difference. Then we compared mean levels of TGF-β in those who were sedated with ketamine and propofol. However, the sampled population was too small and we cannot assume that TGF-β was influenced by any drug administration without a precise knowledge of blood concentration and kinetics of the

drugs at the time of plasma and CSF sampling. Therefore, any assumption on drug interaction is not fully methodologically correct and exhaustive in this setting. The effect of fever was obviously taken into account given its harmful effects on brain metabolism and outcome widely described in literature.

In our NICU we have already described a strategy to contrast fever with low dose diclofenac sodium (DCF). DCF is a hydrophilic NSAID that inhibit the thromboxane-prostanoid receptor, inhibits lipoxygenase enzymes, affects arachidonic acid release and uptake, and activate the nitric oxide–cGMP anti-nociceptive pathway.

In this randomized prospective clinical trial on Twenty-two febrile comatose NCCP, we recorded that the length of time with temperature >38.3 °C was lower in treated patients. Additionally, ICP, cerebral perfusion pressure CPP, mean arterial pressure MAP and heart rate were not affected from this strategy. We consider the population homogenous in light of this strategy and the fever burden was not significative. In our population fever burden was almost abolished because we imped patients top sustain fever and on day 7 (T2) up to 17/19 patients has continuous DCF infusion 75 mg/day. Fever burden was almost abolished and no correlation was possible with TGF-β values.

Seizures

TGF-β expression in CSF varied among patients who had seizures. Generally, TGF-β expression in patients with positive EEG was higher as compared to those who had no positive EEG. However, this is not surprising as the majority of patients had contextually VS. This difference was statistically significant $p<0.05$.

We then split the population of patients who had severe seizure and underwent periods of burst suppression because of continuous non-convulsive activity on continuous EEG. These patients had lower cumulative TGF-β expression in CSF as compared to patients that were less severe.

We speculate that this difference is attributable to the prevalence of other mechanism of injury in these patients, at neuronal basis. Despite non clear differences were noticed in TGF-β in CSF expression and sedation, we focused on the possible effect of different sedation treatment and AEDS (Lacosamide, Phenytoin, Valproate and Levetiracetam) for seizures or status epilepticus. There were no statistically relevant differences in TGF-β expression in CSF. We believe that the number of samples was too small and need to be extended in order to speculate adequate and reliable conclusions on this point.

Hydrocephalous

Permanent hydrocephalous is one of the outmost important complication following SAH. The permanent hydrocephalous shunting procedures are associated to complication as infections and obstruction, therefore it is important to predict which patients will require permanent diversion. Several retrospective studies exist describing prognostic indicators of shunt dependency as female sex, age, aneurysms of posterior circulation, high HH degree and radiological features (high Fisher grade with IVH) and the debate on treatment modality, the clipping versus procedure contribute to HD. Namely the same risk factors known to be associated to worse neurological outcome in SAH.

In the study from Douglas and co-workers, the persistent elevated levels of TGF-β were associated to shunt dependency. In our centre, the timing to definitive shunt is higher and in our population definitive intraperitoneal shunt was placed in 5 patients, four of those were high grade SAH and 1 had SAH from posterior circulation but a good neurological outcome (GOS 4). While in the referred paper, 50% of patients had shunt (10/20). This difference despite the similarity of results in TGF-β expression can be explained by the fact that 5 patients of our cohort had a very good outcome and 3 died within day 14.

3. TGF-β expression and ischemic burden: GOS and documented changes on CT scan (the ASPECTS)

We focused on 2 measure of outcome: a validated scale as GOS and an ancillary new method to look at the residual ischemia with the ASPECTS.

Despite the median levels of TGF-β expression at time 0 in patients with high grade SAH were higher as compared to less severe patients, the median cumulative TGF-β concentration in CSF was not statistically different if compared to GOS. Median values in group of subjects with GOS 4-5 was 17940,68 pg /mL and lower as compared to GOS 2-3 (27565,9 pg/ml). The main reason is that the sample was too small.

In light of our main finding on the temporal changes we tried to generate a cut off to predict VS. We set the value of TGF-β in CSF at 3000 pg/mL as an arbitrary level in between the median values of TGF-β expression in the entire population at To. We found that in those patients who had VS the TGF-β at To was above 3000 in 65% of patients. The presence of values at > 3000 pg/mL TGF-β expression showed sensibility of 81% with a confidence interval of (likelihood ratio) 90% and low specificity to 60% (Bayes Theorem) in patients who developed VS but with $p > 0.05$.

In order to measure in a standardized way the CT scan appearance we decided to use the ASPECTS score despite the several limitations of this methodology. This score is novel, not widely used and with a standard methodology to compare the residual ischemic burden following SAH in a reproducible way between two observers.

During the time course of the disease of the most of our population with sign of VS multimodal monitoring was applied and perfusion CT was performed on clinical judgment in patients who developed VS. MTT values were the main parameter to drive VS management. The decision to apply this score was in the idea to quantify the residual brain ischemia. An expert radiologist, completely blind to the rationale of the study and to clinical records agreed to review all the images and generated an "ASPECTS" trend. Further observation are needed to disclose the impact of prognosis stratification of this methodology.

4. Conclusion, translational relevance and future prospective

This study has several limitations. Results have been restricted to a small number of patients. This fact can be a bias. Ten patients simply dropped out from the study because they died before day

7. That means that we might have lost the more severe. We decided not to include these patients into the analysis because the first aim was to disclose a trend over a seven days period from bleeding. However, in light of the pattern described for the patients who died within NICU a second analysis will be performed in the future to test if a dramatic early increase above 6000 pg/mL can be predictive of unfavourable outcome. If it is the case, a positive result can stratify patients for risk categories and establish that this biochemical marker is capable of predicting the development of brain ischemia and worse neurological outcome and eventually serve as surrogate end point for controlled clinical research studies of the comparative efficacy of interventions designed to optimize cerebral physiology.

The unsolved question is still on the benefit or harmful of TGF-β and if yes at what time. Is TGF-β expression of a reparative process or is it a harmful devastating scenario?

In this study, TGF-β expression was related to severity but not to outcome in those who survived. Is it interesting that patents that had severe cerebral insult because of status epilepticus (YYY) had lower levels of TGF-β as compared to patients with VS but lesser degree of seizure.

Would it be correct a global inhibition of TGF-β or TGF-β signalling to revert VS or prevent DINDs and EBI? The answer should be guided by two factors. First, if further studies will

confirm the preliminary results of this report, then a RCT would be appropriate to test the expression of TGF-β, stratifying patients and the treatment over a longer period.

Second, we concentrated only on one specific pathway of brain damage (inflammation) as part of the pathogenesis of DINDs and EBI. Unfortunately, inflammation can be a double sword.

The final pathway of inflammation is fibrogenesis. And not surprisingly the majority of studies have associated the chronic hydrocephalous to the fibrogenic action of this cytokine.

However, inflammation could equally promote beneficial lesion remodelling and repair. TGFβ1 is a cytokine that is involved in maintaining microvascular integrity through two regulated and balanced processes: endothelial activation demarcated by increased permeability and endothelial resolution, which is characterized by decreased permeability.

In fact, as described in chapter two, the role of TGF-β is controversial in brain disease. Studies on the prevention of Haemorrhagic conversion of the infarctions in murine stroke models showed that bleeding frequently occurred around thin-walled, dilated neovessels in the infarct border zone and was accompanied by decreased expression of transforming growth factor (TGF)-β1 and collagen-4. Injection of TGFβ1 into the lesion border zone greatly reduced infarct bleeding in MO/MP-depleted mice.

In this context, it is widely documented that neuroglia exerts neuroprotection. Microglia are potent regulators of neurogenesis, and in certain settings can promote it. Large body of literature support that the existence of adult neurogenesis can favour recovery and regeneration from many different insults in brain pathologies other than SAH. While the majority of studies have indicated that inflammation is detrimental to neurogenesis, it is now appreciated that the effect of inflammation on neurogenesis is multifaceted and that there are different factors important to regulation of neurogenesis.

Comprehending the signals that regulate adult neurogenesis might harness the potential of neuronal replacement and improve stem cell therapy. It is promising that in neurodegenerative diseases caused in part by microglia, brain-penetrant compounds are under investigation.

How does this study affect therapeutic design to counteract DINDS and EBI?

Harnessing the regenerative capacity of the adult brain is one strategy for repairing and replacing injured tissue, together with enhancing neurotrophic support of existing neurons to promote their survival.

Members of the TGF-β superfamily are known to have pleiotropic effects. Thus, TGFβ1 itself is neuroprotective and anti-inflammatory, it should promote recovery, but it inhibits proliferation of precursors, and also promotes development of

the glial scar through upregulation of many extracellular matrix molecules, and through enhancing the migration of astrocytes. Context dependent activity is the hallmark of cytokine function.

However, future perspectives arise from this latest consideration. In reviewing the literature available on SAH studies it was not fully elucidated if different concentration/expression of TGF-β are associated to opposite effects or if a certain value is associated *per se* to a massive activation of a pathway over another and if massive activation is expression of a dysregulated and not tuned pathway, sign of impending massive EBI. Our preliminary results will be extended in in the continuation of the present study toward two directions. The preclinical evaluation of the role of TGFβ in a mouse model is mandatory for a more exhaustive picture of validity of the role of TGFβ in early and late ischemic damages following SAH.

A larger number of patients will be included and the window frame for plasma and CSF sampling will be set within 21 days from the bleeding in order to track the whole period of VS risk. A second validated biomarker of pure neuronal damage might serve as "internal control" and its expression over time will be measured along with TGFβ.