

Biochemical and genetic markers after subarachnoid haemorrhage

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Cover illustration: Subarachnoid haemorrhage on CT scan, with a giant aneurysm by courtesy of Dr. Hironao Yuzawa, Tohoku University Hospital, Sendai, Japan.

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“A ship rests safely in harbour, but it is not what ships are built for.”
William G.T. Shedd

To my father, who inspired me,
to my mother, who made it all possible
and to my family, who made it all worthwhile

BIOCHEMICAL AND GENETIC MARKERS AFTER SUBARACHNOID HAEMORRHAGE

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ABSTRACT

Background: Subarachnoid haemorrhage is a devastating disease with high morbidity and mortality despite novel treatment options are available. There are no established methods to measure the brain damage occurring due to the bleed and its complications and to predict early neurological outcome of the disease. Genetic predisposition is suggested as one of the determinants of outcome.

Aim: The aim of this thesis was to investigate nine biochemical neuromarkers' course and development in the early phase of aneurysmal subarachnoid haemorrhage (aSAH) with special emphasis on C-reactive protein (CRP) and to test if they could be used as markers of disease progression and possibly long-term outcome. As a tool, we aimed to test a novel multiple biochip array for simultaneous monitoring these markers. Finally, we intended to elucidate the effect of two chromosomes with different genetical polymorphisms on the incidence and development of the disease. (Apolipoprotein E and region 9p21)

Patients and methods: We have consecutively included patients admitted to the Sahlgrenska University Hospital for SAH, where the causative reason was a ruptured intracranial aneurysm. We have recorded the patients' admission status with neurological scales and radiological scores for the severity of the haemorrhage. We collected blood sample for determining genetics and continued to collect serum-samples for biochemical marker detection on day0-4, 6, 8, and finally once on days 11-14. We noted the complication cerebral vasospasm (CVS). A long-term follow-up was performed after one year with detailed neurological examinations. For the genetic studies matching controls were recruited among healthy individuals.

Results: In 98 endovascularly treated patients, we described the pattern of CRP increase after aSAH. It peaked on day3 with a mean value of 53 mg/l and decreased successively without normalising. This pattern was not dependent of infectious status. We noted a difference in increase between the patients with favourable and unfavourable disease development (*i.e.* CVS) and long-term outcome, focal neurology and need of assistance with daily activities (ADL) after one year. In a multivariate regression model with initial neurology, radiological severity, CRP was the only parameter showing significant OR. (OR: 1.25/10 units). We could present a predictive curve for poor outcome in relation to CRP values. Furthermore, we tested a 9 potential neuromarker-containing panel in a test series of 41 patients. Six of these markers, TNFR1, IL-6, hs-CRP, DDMR, NGAL and FABP showed significant correlation to CVS development and different outcome results. Four of the markers (TNFR1, hs-CRP, NGAL & FABP) had moderate or good predictive qualities. In a genetic study, ApoE polymorphism on the 19th chromosome, did not present any effect either on the incidence of aneurysm rupture or CVS development and outcome parameters after aSAH in 154 patients and 221 controls. However we have found a single nucleotide polymorphism (SNP) rs10757278 on the 9th chromosome p21 region, which even after controlling for hypertension and smoking showed a significant negative effect on aneurysm rupture in 183 patient and 366 controls.

Conclusion: CRP proved to be a useful marker for following the course of aSAH and may be applicable for predicting complication or outcome. The tested biochip-neuropanel could be a valuable addition to neuro-monitoring during the initial phase of the aSAH. Finally, not *APOE* polymorphism, but a genetic variant on 9p21 chromosome region affected negatively the risk of aneurysm rupture in West Sweden.

Keywords: *subarachnoid haemorrhage, biochemical markers, genetical markers, outcome*

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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals

- I. Csajbok, L. Z., Nylen, K., Ost, M., Sonander, H., & Nellgard, B. (2015). **In-hospital C-reactive protein predicts outcome after aneurysmal subarachnoid haemorrhage treated by endovascular coiling.** *Acta Anaesthesiol Scand*, 59(2), 255-264. doi: 10.1111/aas.12441

- II. Csajbok, L. Z., Nylen, K., Ost, M., Blennow, K., Zetterberg, H., Nellgard, P., & Nellgard, B. (2015). **Apolipoprotein E polymorphism in aneurysmal subarachnoid haemorrhage in West-Sweden.** *Acta Neurol Scand, Epub. Ahead of publication.* doi: 10.1111/ane.12487

- III. Olsson, S., Csajbok, L. Z., Jood, K., Nylen, K., Nellgard, B., & Jern, C. (2011). **Association between genetic variation on chromosome 9p21 and aneurysmal subarachnoid haemorrhage.** *J Neurol Neurosurg Psychiatry*, 82(4), 384-388. doi: 10.1136/jnnp.2009.187427

- IV. Csajbok, L. Z., Nylen, K., Ost, M., Blennow, K., Zetterberg, H., Nellgard, P., & Nellgard, B. (2015). **Biochip Neuromarker Array, a possible monitoring and prognostic tool after subarachnoid hemorrhage.** *Manuscript.*

POPULÄRVETENSKAPLIG SAMMANFATTNING

Biokemiska och genetiska markörer efter subarachnoidalblödning

Subarachnoidalblödning är en typ av hjärnblödning, som förekommer mellan hjärnan och spindelnäthinnan, utgående ifrån ett brutet pulsåder-bräck på skallbotten och är ett förödande sjukdomstillstånd. Cirka en tredjedel av patienter, som drabbas dör, en tredjedel ådrar dig svåra neurologiska men och en tredjedel återhämtar sig så, att de kan ta hand om sig själva. Det existerar inga metoder att mäta den hjärnskada som blödningen och dess svåra komplikationer åstadkommer samt vi kan inte prediktera sjukdomsförloppet i ett tidigt skede. Man har dock visat, att genetiska faktorer påverkar hur hjärnan återhämtar sig efter sjukdomen.

Ett kroppseget äggvite-ämne, C-reaktiv protein, som hittills använts för att följa infektion i kroppen, kunde vi påvisa att koncentrationerna i blodet av denna följer kroppens hjärnblödningsförlopp tidigt under sjukdomen. Hos hundra blödningsdrabbade patienter mätte vi variationer i nivån av detta protein och observerade olika förlopp mellan de komplikationsdrabbade patienterna med dålig prognos samt de som återhämtade sig bra efter sjukdomen. Utifrån denna skillnad, kunde vi beräkna en prognostisk modell, som så tidigt som två dagar efter blödningen, kunde förutse hur stor risk patienten hade för en dålig sjukdomsprognos ett år efter insjuknandet. Denna prognostiska egenskap var oberoende om patienten blev infekterad eller inte under vårdförloppet.

Vi har dessutom testat en ny biochip styrd mätmetod för 9 olika små proteiner, som man samtidigt kunde analysera från en droppe blod i denna hjärnblödningsdrabbade patientgrupp. Vi ville testa om dessa markörer kunde komplettera eller ersätta de andra mycket farligare mätmetoderna (katetertryckmätning i hjärnan, upprepade röntgenkontroller) som används för att förutspå komplikationer och sjukdomsprognos efter genomgången sjukdom. Vi kunde konstatera att sex av dessa ämnen visade en nära korrelation till den långsiktiga sjukdomsprognosen efter sjukdomen och 4 av dessa kunde med

en stor säkerhet förutspå denna. Dessa fynd bereder plats för ett införande av denna undersökningsmetod på sjukhuset.

Härutöver har vi testat två genetiska variationer på två olika kromosomer och tittat på deras effekter på bristning av pulsåderbräck i hjärnans kärl och komplikation samt sjukdomsprognos efter spindelvävshinneblödning. Den ena variation (Apolipoprotein E), som är känd för sin negativa påverkan på sjukdomsförloppet vid traumatiska hjärnskador och hjärnpropp (stroke) samt vid Alzheimers sjukdom, visade dock ingen effekt på denna typ av blödning. Den andra genetiska variationen, på den 9:e kromosomens p21 region visade däremot en statistiskt säkerställd negativ effekt på kärlets bristningstendens. Dessa studier hjälper oss att bättre förstå de ärftliga faktorer, som påverkar hur våra hjärnor hanterar dessa blödningar.

ABBREVIATIONS

ADL	Activity of daily living	H&H	Hunt and Hess score
ANRIL	Antisense non-coding RNA in INK4 locus	HDL	High density lipoprotein
ApoE	Apolipoprotein E protein	HS	Haemorrhagic stroke
<i>APOE</i>	Human gene coding apolipoprotein E	ICP	Intracranial pressure
aSAH	Aneurysmal subarachnoidal haemorrhage	ICU	Intensive care unit
AUC	Area under the curve	IL-6	Interleukin 6
A β	Amyloid beta	ISAT	International subarachnoid aneurysm trial
BBB	Blood brain barrier	LP	Lumbar puncture
BDNF	Brain derived neurotrophic factor	LR	Likelihood reaction
BMN	Biochemical neuromarker	LT α	Lymphotoxin alfa
CAD	Coronary artery disease	MABP	Mean arterial blood pressure
CDKN2B	Cyclin dependent kinase inhibitor 2B	MAF	Minor allele frequency
CI	Cerebral infarction	MRA	Magnetic resonance imaging angiography
CNS	Cerebrospinal fluid	MRI	Magnetic resonance imaging
CNS	Central nervous system	NCS	Non-convulsive seizures
COPD	Chronic obstructive pulmonary disease	NGAL	Neutrophil gelatinase associated lipocalin
CPP	Cerebral perfusion pressure	NGF	Nerve growth factor
CRP	C-reactive protein	NIHSS	National institute of health stroke scale
CSF	Cerebrospinal fluid	NIVA	Neurointensivvårds avdelning
CT	Computed tomography	NSE	Neuron specific enolase
CTA	Computed tomography angiography	OR	Odds ratio
CVD	Cardiovascular disease	PE	Pulmonary embolism
CVS	Cerebral vasospasm	PNS	Peripheral nervous system
CVS	Cerebral vasospasm	POX	Pulse-oximetry
DCI	Delayed cerebral ischemia	PPV	Positive predictive value
DDMR	D-dimer	RLS85	Reaction level scale
DIC	Disseminated intravascular coagulation	RNA	Ribonucleic acid
DIND	Delayed ischemic neurological deficit	ROC	Receiver operator characteristic curve
DNA	Deoxyribonucleic acid	SAH	Subarachnoidal haemorrhage
DSA	Digital subtraction angiography	SEM	Standard error of the mean
DVT	Deep venous thrombosis	sIL-6R	Soluble interleukin 6 receptor
ELISA	Enzyme linked immunoassay	SIRS	Systemic inflammatory reaction syndrome
EOS	Early onset seizures	SNP	Single nucleotide polymorphism
FABP	Fatty acid binding protein	TCD	Transcranial doppler
FDP	Fibrin degradation protein	TGF β	Tumour growth factor beta
GCS	Glasgow Coma Scale	TNFR1	Tumour necrosis factor receptor 1
GFAP	Glial fibrillary acidic protein	TNF α	Tumour necrosis factor alfa
GFAP	Glial fibrillary acidic protein	WFNS	World Federation of Neurological Surgeons scale
GOS	Glasgow outcome scale		
GOSE	Glasgow outcome scale extended		
GWAS	Genome wide association study		

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INTRODUCTION

I. Background

Aneurysmal subarachnoid haemorrhage (aSAH) is a devastating neurological emergency leaving “one third of the affected patients dead, one third with severe handicap and merely one-third with a good recovery” according to the 1950’s well-known Swedish pioneers of neurosurgery Gösta Norlén and Herbert Olivecrona (Norlen and Olivecrona, 1953). This particular entity of haemorrhagic stroke has been studied in thousands of scientific studies and experiments from Walton’s prognostic description (Walton, 1952) – 15% mortality in the first 24 h, 12% after 1 week, 14% after 2 weeks and 11% after 4 weeks, giving a cumulative percentage of 32% mortality in the first month – until Magni’s study, fairly recently describing a 6 month mortality of 34% (Magni *et al.*, 2015). The overall outcome figures remain surprisingly unchanged throughout the years. From a sceptic’s point of view, no progress has been achieved during the work of two generations. From the optimist’s point of view however, we have come a long way and in the fields of diagnostics, treatment, care and rehabilitation the subarachnoid haemorrhage patients receive an entirely different attention

compared to 60 years ago. The truth lies nevertheless somewhere in between. Although computed tomographic- (CTA), magnetic resonance imaging- (MRA) and digital subtractions-angiography (DSA) are available for diagnostics, micro-neurosurgery with titanium aneurysm-clips and interventional neuroradiology with titanium coils, and titanium-alloy stents are used in therapy approaches, patients are cared for and monitored in specialised neuro-intensive care units and after acute ward, recovery is undertaken in neuro-rehabilitation centres, the outcome after aSAH is still bleak. One could argue, that with better and more efficient ambulance service more patients survive the initial ictus and reach hospital in a worse condition (worse initial neurology), the patients are older and more affected by co-morbidity, and reach therapy centres with intra-ventricular haematomas, which was impossible earlier (Naval *et al.*, 2013). If the outcome-study is controlled for all these parameters, then the outcome after aSAH, has indeed improved (Macdonald, 2013; Grunwald *et al.*, 2014; Naval *et al.*, 2013).

The incidence of aSAH in the world is around 9 of 100,000 individuals, but it has a considerable geographical (Steiner *et al.*, 2013) and socio-economical (Jakovljevic *et al.*, 2001) variation. In Finland and Japan, incidence over 20/100,000 were reported.

This corresponds to a life-time risk of a haemorrhage of 0.5-1 per cent. Many risk factors have been identified in this population cohort (Vlak *et al.*, 2013), and the four strongest are independent in a multivariable model: current and recent smoking (OR: 6.0), hereditary history of SAH (OR: 4.0), hypertension (OR: 2.4), hypercholesterolaemia (OR: 2.0). If age and gender are added to these risk factors, life-time risk can increase up to 7.2 %. A large epidemiological study on the Global Burden of Diseases (GBD 2010) (Krishnamurthi *et al.*, 2014) showed 5.3 million new cases of haemorrhagic stroke (HS) occurring yearly with an overall mortality of 3 million deaths world-wide. They could show a global increase of new HS patients with 47% and an increase of age-standardised incidence of 18.5% but the majority of increase is noted in the low/middle-income countries (LMIC) 86%, where also the 63% of the deaths occurred. In fact, the high-income countries (Europe, N-America and Australasia) could demonstrate an 8% decrease of HS incidence and mortality by 38% in the last two decades. There is some light shining through the darkness though, as even

LMIC demonstrated a reduction in mortality of HS-s by 23%. It is interesting to note that there are 62.8 million disability-adjusted life years lost in the world yearly because of HS and about one fifth is due to SAH. The majority of this life-burden however is placed on the LMIC (86%).

It does not mean, however that HIC spend less money on treatment and rehabilitation of SAH patients. On the contrary, according to the Nationwide Inpatient Sample database, the welfare-system in the USA spends an astounding 2 billion dollars only on acute hospital management of SAH patients. (Hoh *et al.*, 2010) It is nonetheless only a fraction of the total costs associated with these patients as studies of indirect expenditure show an additional 30-97% extra expense for

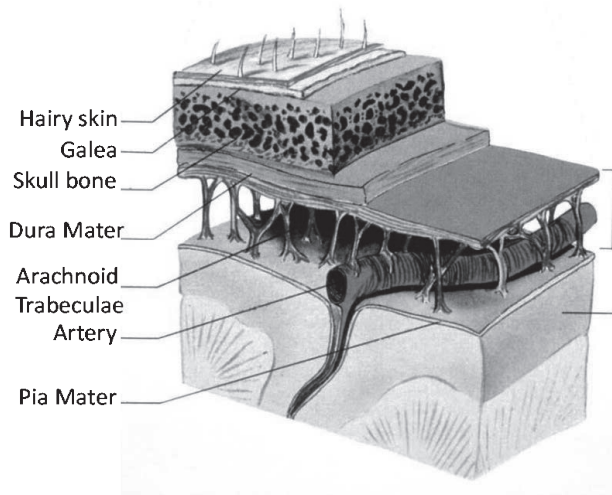


Figure 1. Subarachnoid space with the cerebral cortex. (Adapted from the Univ. of Utah, USA)

productivity loss and informal care (Joo *et al.*, 2014).

A. Mechanism

Subarachnoid haemorrhage is a result of a bleeding from a blood-vessel within the subarachnoid space (Fig 1). The source of the bleeding can be traumatic, from around the injured brain parenchyma (contusion-leak from parenchymal capillaries), venous *e.g.* from the subarachnoid venous network often described as, bridging-veins or perimesencephalic/prepontin bleed, localised to those basal cisterns with possible extension to the suprasellar cistern (Schievink *et al.*, 1994) or arterial from small subarachnoid arteries (malign hypertension bleed) frequently with a parenchymal component. Most often, however (ca. 85%) the subarachnoid haemorrhage originates from the large arteries on the base of the skull, the *circulus arteriosus Willisii* and its branches. (Fig. 2) These haemorrhages have an entirely different disease development and associated with a far more severe outcome. Our studies are dedicated to further explore these types of bleeds.

The largest ever International Study of Unruptured Intracranial Aneurysms – the ISUIA study (Wiebers *et al.*, 2003) – investigated 4060 patients in 59 American,

Canadian and European centres. The localisation and site of the aneurysms were found to be of importance for risk of rupture. The most common sites were the internal carotid artery (CA) 38.3%, middle cerebral artery (MCA) 29.1%, ant. communicating artery (A.Com.A.) and ant. cerebral artery (ACA) 12.3%, post. communicating artery (P.Com.A) 8.5%, basillar tip artery (BA) 7.0%, vertebrobasillar arteries [vert. artery

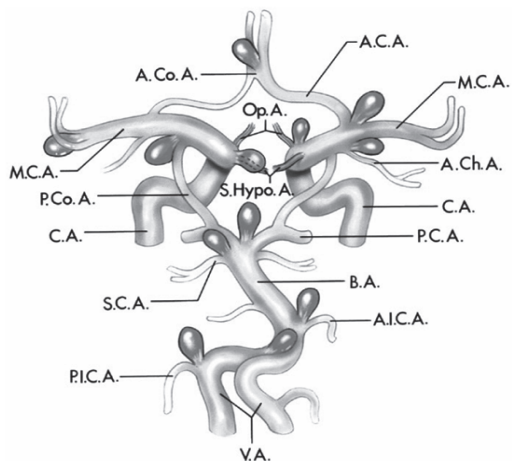


Figure 2. *Circulus arteriosus Willisii* on the base of the skull, the four main supplying arteries to the brain and the most common localisations of intracranial aneurysms. Modified after Rhoton, 2002.

- A.Co.A.: Ant. Communicating Artery
- A.C.A.: Ant. Cerebral Artery (1+2=>12%)
- M.C.A.: Middle Cerebral Artery (29%)
- C.A.: Int. Carotid Artery (38%)
- P.Co.A.: Post. Communicating Artery (8.5%)
- P.C.A.: Post. Cerebral Artery
- B.A.: Basillar:Artery (+ SCA) (7%)
- V.A.: Vertebral Artery (+PICA +AICA)(5%)

(VA), post. inf. cerebellar artery (PICA), post. sup. cerebellar artery (PSCA)] 4.9%.

Altogether the anterior circulation was four times more affected with nearly 80% of aneurysm formations; the potentially more dangerous posterior circulation was involved in 20.4% of cases. Annual rupture rate increased with the size of the aneurysm in the anterior circulation from 0.5% with size 7-12 mm to over 8% if the size were larger than 24 mm. In the posterior circulation it was even larger risk for rupture, it extended from 2.9% (7-12mm) to over 10% in the large ones.

Although the demographical and morphological aspects of aneurysm rupture have been widely investigated, the causative mechanisms are more scarcely debated. One of the main reasons the intracranial vessels behave differently, is because their histological structure is unlike any other vessels in the body. The adventitia, the outer layer of the vessels comprises of connective tissue, vasa vasorum and autonomic nerves, although the cerebral vessels, entering the subarachnoid space change their adventitia to leptomeningeal cells. This way they are bereaved from their elastic limiting shell as they cross the dura mater. The mesothelium is similar in its structure to other arteries, with smooth muscles as main constituent; and the endothelium has pronounced anti-atherogenic, anti-platelet aggregation, anti-adhesion and vasoregulatory properties. (Chalouhi *et al.*, 2012)

Other factors like local trauma (Pereira *et al.*, 2013), infections (Krings *et al.*, 2008), low grade inflammation (Tulamo *et al.*, 2011) and first of all genetic factors (Caranci *et al.*, 2013) have been discussed as causative agents.

B. Diagnosis

The diagnostic investigations after a suspected subarachnoid haemorrhage does not belong among the main topic of this thesis, although as inclusion and exclusion criteria, most of these procedures were mentioned and referred to in the Papers. I decided therefore to give a short summary of the examinations used in the clinical praxis.

1. Xanthochromia

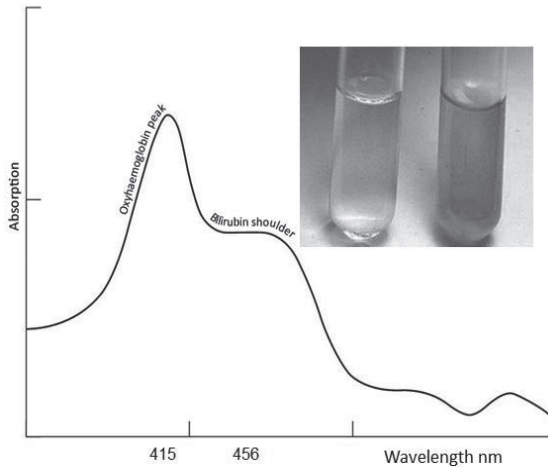


Figure 4 Xanthochromia after subarachnoid haemorrhage. Note the bilirubin absorptions peak, presented as a shoulder at 475 nm.

Lumbar puncture is still performed in the majority of patients presenting with a thunderclap headache and negative CT scan to identify or rule out subarachnoid haemorrhage. The reason is that only 8-12% of patients with sudden headache and no neurological deficit suffer from an aSAH, and 40-50% of aSAH patients are presented with only headache and no other neurological symptoms.(Edlow and Fisher, 2012). For reliable result, the cerebrospinal fluid (CSF) has to be centrifuged immediately to prevent in vitro lysis of red cells with release of oxyhemoglobin. Thereafter spectrophotometry is used to differentiate

the bilirubin absorption on 456 nm wavelength from oxyhemoglobin's 415 nm (Fig 3). The former is pathognomonic for subarachnoid haemorrhage; the latter may be a remnant of a traumatic puncture.(Nagy *et al.*, 2013)

2. CT scan

CT scan is the golden standard for SAH diagnostics. (Fig. 4) The technology of computed scanning is a fast forward motion in development, where the different companies are already 2-3 generations ahead of the current clinical practice. We use currently from 4th to 7th generation CT scanners with fan to cone shape beam and detector array in access of 500 (the latest up

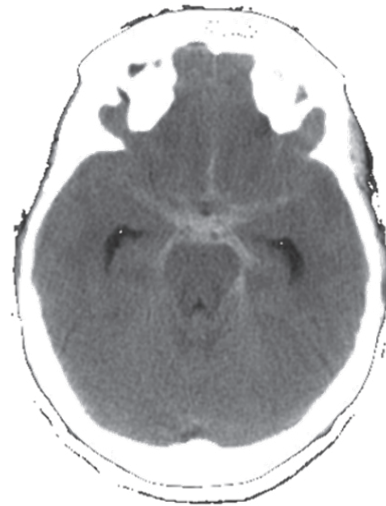


Figure 3. CT scan of a suspected aSAH, with hydrocephalus development. The "crab of death" a typical sign of subarachnoid haemorrhage.

to 2400) in rotational array or in static detectors all around. The 6th generation, so called helical CT with source/detector pairwise rotation and the 7th generation multi-slice CT scanners can give 17 slices per second and is fast enough to examine the heart between beats. Studies started to emerge, which showed that sensitivity and specificity of 3rd generation and newer CT scans are sufficient to diagnose or exclude SAH not later than 6 hours after the onset of symptoms, entirely on the basis of the scan if neuroradiological expertise was at hand. (Backes *et al.*, 2012).

3. CT angiography (CTA)

The latest 5th and 6th generation CT scanners with their speed of scanning and their software interface enabled to perform not only a synchronised contrast X-ray scan, but a high quality 3D reconstruction of the intracranial vessel system, and a first 3D picture of a potential vessel malformation. Many times the quality is good enough to set the diagnosis and initiate the treatment. There are some voices however; speaking out that CT angiography should be used with caution to rule out aneurysm

initially, because of the risk of diagnosing asymptomatic aneurysms instead of a haemorrhage. (Edlow and Fisher, 2012)

4. MRI Angiography (MRA)

Magnetic resonance imaging with magnetic contrast angiography is not an emergency examination of acute subarachnoid haemorrhage investigation. Nevertheless it is a useful method of mapping an unruptured aneurysm before therapy measures are taken, or if neuro-navigational equipment is planned to be used (Fig. 5). Another indication could be severe radiological contrast hypersensitivity, which makes it impossible to perform either CTA or digital subtraction angiography (DSA). The quality of the

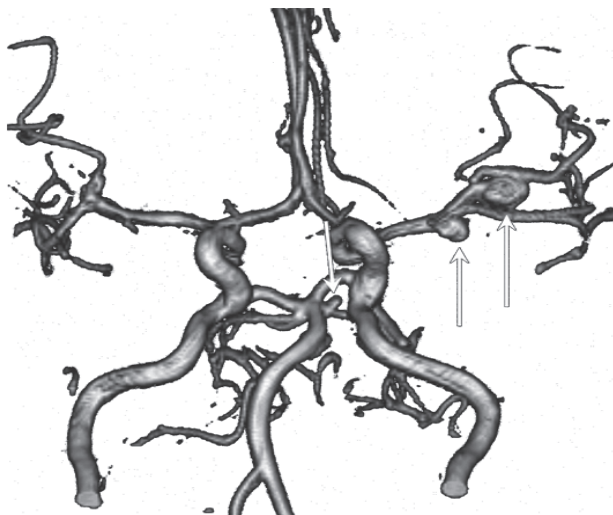


Figure 5. Magnetic Resonance Imaging Angiography of the Circulus Willisii. Three aneurysms are visible.



Figure 6. Bi-plan Digital Subtraction Angiography (DSA) equipment for mapping intracranial aneurysms. (Siemens)

pictures is well in the parity of a medium quality DSA.

5. Digital subtraction angiography (DSA)

It is the most advanced and best mapping possibility of the imaging systems. DSA is rather invasive, as it requires a puncture of a major artery (often the Femoral artery) and a microcatheter which is advanced through the aorta and up to both Carotid arteries by a neuroradiologist. The pictures are obtained with a rotational, often bi-plane X-ray image-taking while a coordinated injection of radiological contrast is performed (Fig.6). One can visualise each and every section of the intracranial vessel-system and perform a

detailed 3D mapping. The patients have to cooperate fully or to be given general anaesthesia in order to achieve the immobility required for the superb quality pictures. Unfortunately the investigation puts some strain on the endothelium of the vessels and burdens the microtubuli of the kidneys with a potentially nephrotoxic contrast agent. It is not uncommon to notice a contrast-

leakage from the aneurysm during the investigation, which is another term for re-bleeding.

C. Treatment

The European Stroke Organisation has recently issued guidelines on the treatment and management of intracranial aneurysms and SAH. (Steiner *et al.*, 2013) Our routines at Sahlgrenska University Hospital consider these guidelines as minimum requirements. The treatment efforts are aimed at three different directions; 1) to prevent re-bleeding 2) to prevent complications 3) to treat complications.

To prevent re-bleeding

It starts immediately after first physician contact of the patient through stabilising his/her condition, ensuring adequate oxygenation, and circulation. If needed, airways have to be secured and artificial ventilation started. Nearly one third of the patients have initial loss of consciousness and one fourth have convulsive seizures. (Fung *et al.*, 2015) It is imperative to cease these seizures to continue the patient management. To establish monitoring is fundamental as oxygenation, circulatory stability and neurological assessment are determinant factors in therapeutical decision-making. Invasive blood-pressure monitoring, plethysmographic pulse-oximetry (POX), ECG monitoring, urinary output and neurological valuation are minimum monitoring standards even during transport to tertiary (neurosurgical) therapy centres. Systolic arterial pressure should be kept under 180 mmHg, but should not be lowered to more than to a mean arterial pressure (MAP) of 90 mmHg. (Steiner *et al.*, 2013).

Tranexamic acid (Cyklokapron® i.v. 1g three times daily), a fibrinolysis inhibitor is recommended in our centre as a pharmacologic re-bleeding prophylaxis, given directly after the diagnosis is

established. (Hillman *et al.*, 2002) This treatment is continued until the aneurysm is secured.

The efforts of re-bleeding prevention continue in the neurosurgical department, where the aneurysm is mapped with DSA and a 3D image reconstruction is created. After a discussion between the neurosurgeon and the interventional neuroradiologist, a joint decision is made how to secure the aneurysm.

1. Surgical clipping

One of the options is to use an intraoperative method, involving an open craniotomy and

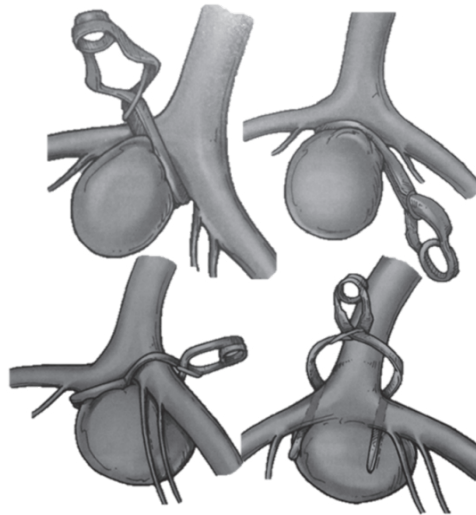


Figure 7. Titanium surgical Sugita clips for different aneurysm applications

surgical exploration of the aneurysm. It is a major neurosurgical operation when a special clip, first used by Walter Dandy at the Johns Hopkins Hospital in Baltimore, 1937 and

refined by Kenichiro Sugita at the Nagoya University in the middle of the 1970-s (Sugita *et al.*, 1984), is applied to the neck of the aneurysm, thereby obstructing the flow to it (Fig. 7).

2. Endovascular coiling

Endovascular treatment of an aneurysm begins the same way as a DSA, *i.e.* a puncture in one of the femoral arteries and navigating up a catheter via the aorta into the carotid/vertebral arteries and in this case a microcatheter further to the proximity of the aneurysm. Through this microcatheter one can fill the cavity of the aneurysm with flexible detachable platinum coils designed

named Guglielmi Detachable Coil (DGC®) and following the FDA's approval in 1995, more than 140 different versions, coatings and application-platforms are manufactured. The procedure was a major step forward in micro-invasive vascular neurosurgery. Depending on the centres routines and competence endovascular coiling represents now between 50-80% of all aneurysm treatment modalities. A few years later, in the beginning of the 1990s, the same institute in the UCLA presented micro-stents in combination with coiling (Turjman *et al.*, 1994). It allowed treating even the wide-necked aneurysms, which previously had solely been the neurosurgeon's domain (Fig.8).



Figure 8. Combined stent, coil and surgical clip in a complicated aneurysm case in the Mayfield Clinic, Cincinnati, Ohio

3. Conservative treatment

A few patients, approximately 2 - 4 % of the admitted subarachnoid haemorrhage cases do not undergo active neurosurgical/interventional treatment because of an accumulation of encumbering circumstances *e.g.* extremely high age, poor neurological income status (H&H - 5, WFNS - 5, GCS < 5) and/or other severe comorbidity where general anaesthesia would deteriorate their condition. These patients receive basic

by Guido Guglielmi in the 1980s. (Guglielmi *et al.*, 1992) After his concept the coils are

intensive care with respiratory and circulatory support in addition to fluid and electrolyte management, but their aneurysm(s) are left untreated.

D. Complications

The complications after aSAH can be divided into early and late ones. The collective name for the early complications is “early brain injury”, and it occurs within the first 72 hours after the haemorrhage. It is a direct result of the bleed and has a strong association with the amount of extravasated blood and the initial rise of intra cranial pressure (ICP). Some of the potential mechanisms are discussed in a recent article (Rowland *et al.*, 2012) and characterised as mechanical (constriction from bleed, cisternal blood, hydrocephalus), physiological (elevated ICP, reduced CPP, impaired cerebral autoregulation, vasoconstriction), ionic (cortical spreading depression, impaired Ca^{2+} homeostasis, K^+ efflux, Mg^{2+} disturbance), inflammatory (NO-synthetase activation, endothelin-1 release, oxidative stress, platelet activation) and cell death derived (apoptosis and necrosis of endothelium, neurons, astrocytes). Although these mechanisms have started before the late complications occur, it is reasonable to think that they have an impact on the likelihood and severity of these late difficulties.

1. Cerebral vasospasm (CVS)

CVS in the literature denotes radiological vasospasm and it includes Trans-Cranial Doppler (TCD) identified vasospasm as well, which is a common way of following this complication in the ICU. It also comprises naturally angiography-verified vasospasm with CTA, MRA or DSA. As CVS, has been associated with late neurological complications, it is a frequently used marker in genetic and biochemical signal studies.

2. Delayed cerebral ischemia (DCI)

Recently, a multidisciplinary research group defined this entity (Vergouwen *et al.*, 2010), as there has been a great confusion in the definition and characterisation of this important complication. In American literature, one might find it as Delayed Ischemic Neurological Deficit (DIND) (Lai and Du, 2015). It covers focal neurological impairment or a decrease of at least 2 points in consciousness measured by Glasgow Coma Scale (GCS). It is an extremely important complication, as DCI is the most prominent cause of mortality between postictal day 3 and day 14. (Rowland *et al.*, 2012) The majority of the above mentioned “early brain injury” mechanisms have been claimed to play a role in this deleterious complication.

3. Cerebral infarction (CI)

CI is defined as radiological (CT, MRI) signs of infarction within 6 weeks after an aSAH, the latest CT prior to death (in 6 weeks) or an autopsy verified infarction. These signs should not be directly connected to operation or embolisation. These radiological signs though must not be present within 48 hours of the bleeding (Vergouwen *et al.*, 2010). To be more confusing, in American literature this could be named as DCI, in contrast to older infarction or infarction directly related to treatment (post-operative or post-

reason why all aSAH patients receive iv. or oral nimodipin, during 10-14 days after the bleeding.

II. Neurological, radiological assessment

A. Admission assessment

1. Hunt and Hess scale

SAH patients’ early evaluation has been advocated from the early 1950’s (Norlen and Olivecrona, 1953) and a classification has been systematically used since Botterell published his article on assessment of the perioperative risk of SAH patients.

Hunt & Hess grade	Properties	Approx. survival rate
Grade 1	Asymptomatic, minimal headache, slight neck stiffness	70%
Grade 2	Severe headache, neck stiffness, no neurologic def. except cranial nerve palsy	60%
Grade 3	Drowsy, minimal neurological deficit	50%
Grade 4	Stuporous, moderate to severe hemiparesis, early decerebrate rigidity, vegetative disturbances	20%
Grade 5	Deep coma, decerebrate rigidity, moribund	10%

Table 1. Hunt and Hess grading scale and expected rate of survival at the time of publishing(Hunt and Hess, 1968)

embolisation complication). It is understandable, that review articles and meta-analyses have problems defining the end-points of the studies.

Apart from an active neurosurgical management and optimised neuro-intensive care, the only drug which is documented to improve outcome is nimodipin. This is the

(Botterell *et al.*, 1956) From his five-grade scale evolved the most used SAH grading scale developed by William Hunt and Robert Hess from Ohio and was the standard assessment instrument for half a century. (Table 1) (Hunt and Hess, 1968). As it has been used world-wide and extensively validated, we have chosen this instrument in

most of our papers for assessing SAH severity at admission.

2. Glasgow Coma Scale (GCS)

As different kinds of neurological emergencies started to be admitted to dedicated Emergency Units, Teasdale and

Sweden, an easier-to-use 8 graded motor responsive scale has gained popularity. (Starmark *et al.*, 1988) Grades 1-3 describe conscious patients, while in Grades 4-8, the patients are unconscious. This grading is widely used in prehospital and primary trauma/neuro-emergency assessment. (Table

3.)

GLASGOW Best motor respons	COMA Best verbal response	SCALE Eye opening
Obeys commands (6)	Oriented speech (5)	Spontaneous (4)
Localises pain (5)	Confused speech (4)	To command (3)
Flexor withdrawal (4)	Words only (3)	To pain (2)
Abnormal flexion (3)	Sounds only (2)	None (1)
Extension (2)	None (1)	
None (1)		

Table 3 Glasgow Coma scale for assessment of consciousness and responsiveness (Teasdale and Jennett, 1974)

Jennett have realised the importance of an aetiology-independent grading scale (Teasdale and Jennett, 1974) and introduced a behavioural assessment grading based on the best motor, verbal response and eye opening, awarding points for each activity. The total sum of the points, (max.: 15, min.: 3) provide the GCS. (Table 2) This scale has since then been used for assessment of altered consciousness of all possible causes in the emergency departments.

3. Reaction Level Scale 85 (RLS85)

In the Nordic countries and especially in

4. World Federation of Neurological Surgeons scale (WFNS)

Having experienced the shortcomings of the Hunt and Hess scale a task force within the largest community of neurosurgeons worked for years to establish a more user friendly, practical, validated and widely

Reaction Level Scale 85
1. Alert
2. Drowsy or confused
3. Very drowsy or confused/agitated
4. Localises pain
5. Withdrawal movements
6. Stereotype flexion movements
7. Stereotype extension movements
8. No response to pain

Table 2. Reaction Level Scale 85 a responsiveness grading for fast neurological assessment. (after Starmark, Stålhammar *et.al*)

WFNS	GCS	Major Focal deficit
I.	15	Absent
II.	14-13	Absent
III.	14-13	Present
IV.	12-7	Absent / Present
V.	6-3	Absent / Present

Table 4. World Federation of Neurological Surgeons scale for assessment of subarachnoid patients (Teasdale et al., 1988)

accepted scale for initial neurological evaluation of SAH patients. (Teasdale et al., 1988) They adapted their scale to an already established responsiveness assessment scale, the GCS and added the existence or absence of major focal deficit. They finally agreed on a 5 graded scale with a combination of these factors (Table 4). A problem arose however, when patients presented with different levels on different axis of the scale i.e. intact cortical function but major focal deficit. Patients in those cases received the worse of grades. This is one of the reasons why several modifications of WFNS scale have emerged recently (Sano et al., 2015; Naval et al., 2014).

5. The Fisher scale

As radiological diagnostics of suspected SAH in patients became more important, Fisher realised the significance of a validated scale based on the distribution of blood visualised on the initial CT examination (Fisher et al., 1980). The scale was originally intended to help predicting those patients at risk for cerebral vasospasm, but it was early connected to outcome (Gilsbach et al., 1988)(Table 5).

There are several limitations of this 4 grade-scale; i.e. it does not differentiate between intra-ventricular and intra-parenchymal blood, it is a blunt instrument with only 3 grades where blood at is all visible and there is temporal course of the blood distribution in the

Fisher scale	Subarachnoid blood
1.	None
2.	Diffuse, thin (< 1mm)
3.	Clot or thick (\geq 1mm)
4.	Diffuse/none, Cerebral/Ventricular blood,

Table 5. The Fisher scale for radiological evaluating subarachnoid haemorrhage on CT scan

subarachnoid/cisternal/intra-ventricular space, thereby a time-limit should be set for this grading. Similar criticism and a demand for new scales came up in the early 2000s (Smith *et al.*, 2005) and led to different modifications of the original Fisher scale (Claassen *et al.*, 2001; Frontera *et al.*, 2006). As these revised scales were not available at the time of study-design, we employed the original, which was nevertheless a validated, widely-used assessment-method.

B. Outcome assessment

The strength of the present studies and our project lies in the outcome assessment. We planned for long-term follow up, which we considered as a minimum of one year. We hypothesized, what later research strengthened (Wilson *et al.*, 2013) that a substantial recovery may occur beyond the usual follow-up period of 3 - 6 months. At the time of our study design, there were not many large aSAH patient -groups, that were followed over such long period without patient loss. Furthermore, we have utilised the most sophisticated grading scales available at the time and tried to capture all aspects of a possible handicap *i.e.* global, focal neurological, psychological

dysfunctions and functioning in daily life. As recommended (Anderson *et al.*, 1993), we had to choose an outcome investigator not involved in the patients' care. As these complex scaling systems required a neurological specialist to examine the patients and our intention to minimise the inter-examiner variability ensued that only one neurologist performed all the follow-up examinations. My never fading gratitude goes to my co-author and co-worker, Karin Nylén MD, PhD for this demanding task. I give bellow a brief summary of the scaling instruments utilised.

1. Glasgow Outcome Scales (GOS, GOSE)

GOS is the most widely used outcome evaluation tool, where patients are allocated

GOSE	GOS	Description
1.	1.	Dead
2.	2.	Vegetative state
3.	3.	Severe disability lower
4.	3.	Severe disability upper
5.	4.	Moderate disability lower
6.	4.	Moderate disability upper
7.	5.	Good recovery lower
8.	5.	Good recovery upper

Table 6. Glasgow Outcome Scale (GOS) and its extended variant (GOSE) for outcome assessment

to a category on a 5 or in the extended version (GOSE) 8 hierarchical categories during an interview (Teasdale and Jennett, 1974). This relatively easy-to-use scale gives a general index of overall outcome and it reflects disability, rather than impairment compared to pre-morbid status; *i.e.* how the handicap affects functioning in major areas of life (Table 6). It allows comparison between different patient-groups (Marschall, 1987) and been suggested as a measure of outcome in clinical trials (Clifton *et al.*, 1992). The questions are based on the areas

of everyday life *e.g.* independence at home, independence outside home, employability, engaging in pre-morbid social and leisure activity and interpersonal relationship (Jennett *et al.*, 1981). If inter-observer variability is to be kept at minimum a structured questionnaire-based interview with few, educated raters have to be employed (Wilson *et al.*, 1998). We used the questionnaire for the extended GOS (GOSE) to allow a wider differentiation of the data.

Category	0 point	1 point	2 points	3 points	4 points
1a. L. of consciousness	Alert	Drowsy	Stuporous		
1b. LOC questions	Both correct	One correct	Incorrect		
1c. LOC commands	Obeys both	Obeys one	Incorrect		
2. Best gaze	Normal	Partial palsy	Forced deviation		
3. Visual field	No loss	Partial hemianopia	Compl. hemianopia	Bilat. hemianopia	
4. Facial paresis	Normal	Minor	Partial	Complete	
5a. Motor arm left	No drift	Drift	Cannot resist gravity	No effort against grav.	No movement
5b. Motor arm right	No drift	Drift	Cannot resist gravity	No effort against grav.	No movement
6a. Motor leg left	No drift	Drift	Cannot resist gravity	No effort against grav.	No movement
6b. Motor leg right	No drift	Drift	Cannot resist gravity	No effort against grav.	No movement
7. Limb ataxia	No ataxia	Present in one limb	Present in two limbs		
8. Sensorium	Normal	Partial loss	Severe loss		
9. Best speech	No aphasia	Mild-mod. aphasia	Severe aphasia	Mute	
10. Dysarthria	Normal articulation	Slurring of words	Unintelligible		
11. Neglect	No neglect	Partial neglect	Complete neglect		

Table 7. National Institute of Health Stroke Scale (NIHSS), instrument to measure focal neurological deficit

2. National Institute of Health Stroke Scale (NIHSS)

NIHSS is a focal neurological deficit assessment scale which was developed by Brott and co-workers (Brott *et al.*, 1989) to document impairment after a stroke. It has been widely accepted as a study instrument, and is recommended by the American Heart Association for outcome classification even in clinical use. It assigns points for focal neurological deficits on an increasing scale in the areas of language, speech, coordination, visual field, neglect, eye-movement, consciousness and motor- and sensory functions (Table 7).

3. The Barthel Index

The Barthel index is a measure of independence in 10 important activities of daily living (Table 8). It has been a valuable instrument to estimate the amount of

assistance a person requires after an illness or injury. It was initially designed to monitor improvement after treatment in patients with chronic neurological diseases (Mahoney and Barthel, 1965). It measures the functional capacity in eating, bathing, dressing, walking

Activity	0 points	5 points	10 points	15 points
Feeding	Unable	Needs help	Independent	-
Bathing	Dependent	Independent	-	-
Grooming	Needs help with personal care	Independent	-	-
Dressing	Dependent	Needs help, but manages half	Independent	-
Bowels	Incontinent	Occasional accident	Continent	-
Bladder	Incontinent	Occasional accident	Continent	-
Toilet	Dependent	Needs help	Independent	-
Transfer (bed-chair)	Unable	Major help	Minor help	Independent
Mobility	Immobile	Wheel-chair independent	Walks with one pers. aid	Independent
Stairs	Unable	Needs help	Independent	

Table 8. The Barthel Index. Assessing Activities of Daily Living (ADL), a measure of independence

and getting out of bed and chair. The points in the index give a good estimate on how much nursing assistance a person requires. On the contrary, patients achieving full credit in the Index, are not necessarily capable of living an independent life.

C. Physiological parameters

To keep physiological parameters within normal limits is important in any disease's therapeutic regime, notwithstanding it is even more important in aSAH, when the central coordination of the different mechanisms is failing. Comprehensive therapeutic guidelines for the management of SAH patients in the Neurointensive Care setting were published a few years ago (Wartenberg, 2011; Smith, 2007) summarising the main aspects of the treatment protocols. I describe our intentions regarding the physiological limits, at the time our study-patients were treated in the NICU, Sahlgrenska University Hospital.

Haemoglobin >120 g/l,
S-sodium >135<150

mmol/l, S-potassium 4.0-5.0 mmol/l, S-albumin 35-50 g/l, core temperature 37 ± 0.5 ° C, MABP between 70-100 mmHg, intracranial pressure (ICP) <20 mmHg, Cerebral perfusion pressure (CPP = MABP-ICP) >60 mmHg, pO₂ 12-18 kPa, pCO₂ ca 4.5 kPa and normalized pH. Blood glucose was kept stringently between 4-6 mmol/l.

III. Genetic neuromarkers

1. Apolipoprotein E (ApoE)

Apolipoprotein E (ApoE) is the protein-part of very low density lipoprotein (VLDL) group, which are often remnants of chylomicrons. The protein is only 299 amino-acid long and circulates in blood, cerebrospinal fluid (CSF) and central nervous

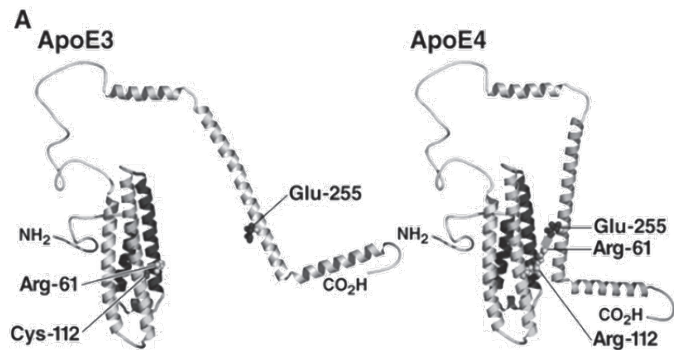


Figure 9. Apolipoprotein E3 and E4's 3D structure. Note the difference in mobility of the arm, and thence difference in function! With permission. (Mahley and Huang, 2012)

system (CNS) tissue interstitial fluid (Fig.9). In the CNS, it is produced by the astrocytes and one of its functions is to transport cholesterol and other lipids through membranes, thereby ApoE is directly responsible for the clearance of plasma lipoproteins from the cell. The mechanism is that ApoE serves as a critical ligand to low density lipoprotein receptors. This function, to redistribute lipoproteins into the cells is

fundamental for cellular reparatory processes (Mahley and Huang, 2012).

The protein is coded by a single gene locus on the 19th chromosome's short arm (19q13.2) and there are three different alleles ($\epsilon 2$, $\epsilon 3$, $\epsilon 4$,) encoding three different isoforms of the protein (E2, E3, E4) with profoundly different form and function. (Mahley, 1988)

ApoE was early associated with Alzheimer disease (Mahley, 1988; Blennow and Cowburn, 1996; Strittmatter *et al.*, 1993), and this was the start of an intensive research on its neuro-pathological effect. Kim has reviewed some of these known mechanisms (Kim *et al.*, 2009); impaired neurite outgrowth, cytoskeletal disruption, mitochondrial dysfunction, impaired synaptogenesis, amyloid (A β) production, altered clearance and deposition, lysosomal leakage and apoptosis and finally impaired learning in rodents. The discovery of these detrimental effects led to further research within all possible neurological disorders, among which traumatic brain injury (Helgadottir *et al.*, 2008), multiple sclerosis (Fazekas *et al.*, 2001), stroke and intracerebral haemorrhage (McCarron *et al.*, 1999), fronto-temporal dementia (Agosta *et al.*, 2009) and Parkinson disease (Martinez *et al.*, 2005) are found to be negatively affected by the presence of ApoE4.

SAH patients have also been investigated for ApoE effect, but the results have been rather incongruent. About equally as many have found a negative effect of ApoE4 (Niskakangas *et al.*, 2001; Leung *et al.*, 2002) as no effect at all (Morris *et al.*, 2004; Fontanella *et al.*, 2007). This ambiguity of the results and the regional relevance, as there is a geographical difference in allele distribution in the world, which motivated our study II.

2. Chromosome 9p21

Genetic association with SAH has been recognised for years as it was well known that first degree relatives to patients with intracranial aneurysm (IA) or SAH have 3-5 times increased risk to develop the same disease. (Gaist *et al.*, 2000) and again in the same relatives the risk of harbouring an unruptured aneurysm has grown to 9%, that

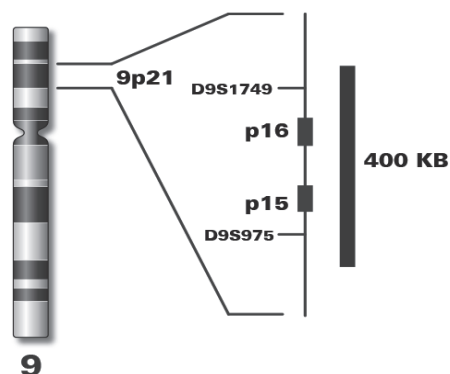


Figure 90 Chromosome 9 and segment 9p21's localisation and approx. size. With permission from Leica Microsystems AB, Bromma, Sweden.

is 4.5-time-increase of risk compared to the general population. (Ronkainen *et al.*, 1997)

These family-linkage studies and large genome-wide association studies (GWAS) identifying a locus on the 9th chromosome's short arm (9p21) as risk area for IA (Bilguvar *et al.*, 2008) and even other blood-vessel related anomalies, like coronary artery disease (CAD), aortic aneurysm, and arterial stiffness encouraged further investigation in the region to search for the responsible genetic variant (Fig.10). As no causative genotype had been identified on this location at that time, we had to further narrow down the pursuit to single nucleotide polymorphisms (SNPs) – one single base-pair interchanges, which are the most frequent occurring genetic alterations. We have investigated in Paper III, if any of the identified SNPs correlate to ruptured IAs independently from other risk factors.

IV. Biochemical neuromarkers

1. C-Reactive Protein (CRP)

CRP was discovered by Tillett and acquired its name from a reaction with the C-

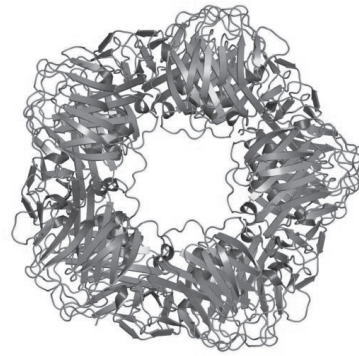


Figure 10. C-Reactive Protein with its 5 sub-units organised in an annular, pentameric form. (Public domain)

polysaccharide of Pneumococcus (Tillett and Francis, 1930). It is one of the body's most important acute-phase proteins mainly synthesised in the liver. It responds to cytokines, mainly IL-6, secreted by macrophages, T-cells and also adipocytes. It has an annular form and belongs among the pentameric proteins, which are common as ionic-channel receptors or viral capsids. They can form a channel, expressing receptors and with their ligand attached, they often undergo a conformational change, thereby interacting with the molecule (often neutralising, or facilitating transport). It conforms to its putative roll to detoxify and neutralise harmful substances escaping into the circulation (Fig.11).

As an acute-phase protein, CRP's concentration rises quickly and can increase well over 1000-fold and peaks around 48 h after the insult and returns to base-line after

7-12 days if the stimulus is removed (Rothoerl *et al.*, 2006). It is cleared from plasma monoexponentially, independent of concentration with a half-life of ~19 h and it makes the production the only determinant of its level. This makes CRP a perfect marker of inflammatory activity in the body.

Numerous publications have connected SAH to inflammatory mechanisms (Rothoerl *et al.*, 2006; Bhardwaj, 2003; Juvela *et al.*, 2012), but all of them included surgically treated SAH patients, thereby clouding the inflammatory mechanisms caused by SAH with the one induced by a craniotomy (Mirzayan *et al.*, 2007). It was plausible to think that merely endovascularly treated patients' CRP response would have a closer relationship to the haemorrhage and therefore the outcome. We explored this hypothesis in Paper I and we returned to CRP in Paper IV. The biomarkers listed below were included in the Neuropanel biochip array we tested in SAH patients in Paper IV.

2. Interleukin 6 (IL-6)

Interleukin 6 is a pro-inflammatory cytokine (secreted protein, signal molecule) and it is one of the founding members of a now-a-days rather large group of interleukins which were named after being described first in leucocytes. They are glycoproteins by chemical nature, consisting of 170-180 amino-acids and show a quadruple helix

bundle structure. Prior to a consensus meeting in Switzerland in 1979, it was called B-cell stimulatory factor 2 (BSF2) or Interferon β 2 because of its first described effect on B-cells' differentiation to immunoglobulin secreting cells. Besides this immunogenic effect, IL-6 is now accepted as neuropoietin (Erta *et al.*, 2012) consequent to its neuro-protective and neuron differentiating effect. It has an important role as acute-phase reactant from hepatocytes, acting as primary activator of CRP. IL-6 is activated in infectious and inflammatory diseases, autoimmune processes, in diabetes, atherosclerosis, depression, Alzheimer - disease, rheumatoid arthritis and many other conditions. It is produced in macrophages, Th2 cells, B-cells, astrocytes, microglia, neurons, endothelium and hepatocytes. The major neuro-protective effect comes from its attribute that it inhibits $TNF\alpha$ and $IL-1\beta$ via activating $IL-1ra$ and $IL-10$. IL-6 also contributes to neuro-regeneration by promoting neuro-remodelling, and angiogenesis (Gertz *et al.*, 2012). Finally, it has to be mentioned, that IL-6 is the main regulator of fever in the acute-phase response by crossing the blood-brain barrier and promoting prostaglandin E_2 synthesis in hypothalamus thus changing the body temperature.

3. Tumor Necrosis Factor Receptor 1 (TNFR1)

TNFR1, together with TNFR2 are receptors for tumour necrosis factor α (TNF α) and lymphotoxin α (LT α), both pro-inflammatory cytokines and they are expressed on most cell types in the body. TNF α and LT α play a major role in immune regulation and in host-defence reactions. They are expressed mainly by macrophages after cell injury or inflammation but other haematopoietic or non-hematopoietic cell-types can be involved. (Probert *et al.*, 2000) TNF α is responsible for recruitment of leucocytes to inflammation area and regulates cell-death by proliferation, cytotoxicity and apoptosis. (Wallach *et al.*, 1997) The majority of biological response to TNF α manifests through TNFR1 receptors, while TNFR2 has some special functions. It mediates T lymphocyte proliferation and certain suppression of inflammatory processes (Peschon *et al.*, 1998). TNFR1 receptor mediates endotoxin shock and overexpressed TNF α / LT α or TNRF1 is detrimental, resulting in rheumatoid arthritis or multiple sclerosis. TNF α / LT α 's pro-inflammatory properties demonstrate local inflammation in transgenic mice and their cytotoxic effects can trigger apoptosis and cell-death in oligodendrocytes. (Selmaj and Raine, 1988).

TNF α studies are however contradictory and it may depend on the short half-life of this cytokine, and that it can bind to undetectable complexes (Beutler *et al.*, 1985). It has been suggested that the soluble forms of TNFR1 and 2 with their longer half-life are more reliable indicator of the TNF system activation than the short-lived cytokine itself (Kreuzer *et al.*, 1996). Moreover the soluble TNFR1 can be viewed as an independent inflammation marker as its transition to soluble isomer is a complex activity, dependent on other inflammatory mechanisms (Diez-Ruiz *et al.*, 1995).

4. Neutrophil Gelatinase Associated Lipocalin (NGAL)

NGAL or Lipocalin-2 is a 25 kDa glycoprotein and has its principal function in innate immunity where it concentrates and sequesters iron in the form of siderophores. This defence mechanism is pivotal in bacterial protection (Yang *et al.*, 2002). It was first described in neutrophils, but now nearly all other cell-types have been shown to express NGAL under different circumstances *e.g.* in the brain under oxidative stress (Naude *et al.*, 2012).

It is widely used as marker for acute kidney injury and a general inflammation signalling substance. During inflammatory processes the expression of NGAL is altered *i.e.* in meningitis, myocarditis, psoriasis,

rheumatoid arthritis. The serum level of NGAL is considered low below 20 ng/ml, medium round 200 ng/ml and high above 1200 mg/ml. Apart from renal injury detection and delayed graft dysfunction (Shapiro *et al.*, 2010), it has been shown to predict septic shock (AUC:0.77), and death (AUC:0.79) among SIRS patients in the ICU, especially together with IL-1 and Protein-C. The cut off level was 48 ng/ml (Endre *et al.*, 2011). Further, it was found useful in heart failure, epithelial malignancies and even in late-life depression (Naude *et al.*, 2013). The proposed mechanism of NGAL in the CNS encompasses cellular-stress via increased expression of NGAL, leading to microglia activation, astrogliosis and neuronal apoptosis, resulting in behavioural changes (Gouweleeuw *et al.*, 2015). These properties qualify NGAL to be included into a cerebral injury-marker panel (Paper IV).

5. Glial Fibrillary Acidic Protein (GFAP)

GFAP is a type III intermediary filament, with an average diameter of 10nm (Fig.12). It is found in glial cells in the CNS and is responsible for the cytoskeletal structure of astrocytes, helping to maintain their mechanical strength. Apart from structural maintenance, GFAP plays a role in cell communication and even in the functionality of the

blood-brain barrier (BBB). During mitosis, they regulate the filament network in the cytoplasm controlling the cell partitioning. In astrocyte-neuron interaction, GFAP may have a bridging role through the glymphatic system and this may also be the way it reaches the bloodstream via venous adjunctions or when BBB disruption occurs (Plog *et al.*, 2015).

Following brain injury and in some chronic diseases the astrocytes respond with an unspecific transformation called astrogliosis. It is a proliferation, hypertrophy and building of abundance of intermediate filaments. The severity and time-course varies in this transformation from slowly and sporadically as in multiple sclerosis or massive and fast-progressing, as in fibrinoid leucodystrophy, Alexander's disease.

If GFAP's level in the astrocytes is reduced (genetic disorders, transcriptions difficulties, multifactorial), this may lead to other neuropsychiatric syndromes, like schizophrenia



Figure 11. Glial Fibrillary Acidic Protein (GFAP). Intermediary filaments form a quaternary chain.

and depression. The serum levels are normally undetectable if not an immense disruption of BBB occurs *e.g.* SAH, cerebral trauma, intra or cerebral haemorrhage (Mayer *et al.*, 2013). In that case the concentration is closely associated with the injury size (Nylen *et al.*, 2007; Nylen *et al.*, 2006; Nylen, 2007).

6. Brain-Derived Neurotrophic Factor (BDNF)

BDNF is a signal protein and one of the most prominent members of the neurotrophins or nerve growth factors. Its closest relatives in this family are the neurotrophins 3 and 4 and nerve growth factor (NGF). It is synthesised in the endoplasmic reticulum and passed on into vesicles in the brain and the periphery, like in retina, kidneys saliva and the prostate. It acts on the neurons, promoting neuronal survival, neurogenesis and synaptogenesis. (Lu, 2003) In animal studies it reduces ischemic injury and improves recovery and post-injury regeneration (Almeida *et al.*, 2005).

BDNF is most active in the cortex, hippocampus, basal ganglia and areas, where learning, memory and higher thinking take place. It may work even in adult brain through neural stem-cells, by promoting neuroneogenesis (Pencea *et al.*, 2001). In knock-out mice, it produces severe brain developmental defects and even perinatal

death. This emphasises the importance of this neurotrophin in the embryonal brain formation and neuronal growth. Normal serum level was reported round 30 ng/ml and low values were considered around half that level. It is easy to understand why this neuromarker is included in a neuropanel-array.

7. Fatty-Acid Binding Protein (FABP)

Lipids, where fatty-acids, eicosanoids and retinoids are included function not only as fuel source, building-blocks to membranes and cellular structures, but also as intra- and extra-cellular signalling substances. Recent studies, summarised in a review (Hotamisligil and Bernlohr, 2015), enlighten us that lipids take part (i) in modifying the actions and locations of proteins, such as kinases or ion-channels; (ii) signal through proteins to cell surface or between cells as G-protein; (iii) ligand for transcription factors, modifying expressions of other regulators; (iv) regulate hormone actions, like PI3K, and NFκβK pathways and finally (v) pattern recognition receptors.

The insolubility and toxic effects of the free forms of these molecules, require, however a type of non-catalytic binding-protein *e.g.* FABPs. The original FABP was described in small intestine as a 12 kDa intracellular protein (Ockner *et al.*, 1972) but since then

similar molecules were shown in kidneys, adipose-tissue, myocardium, liver and brain. Their function were further elucidated and revealed that they not only buffer and physically transport lipids, but they serve as mediators and as communicating agents within and between cells and between organs thus supporting immunometabolism. In the CNS, 4 isoforms are detected B-(brain), H-(heart), E-(epidermal) and M-(myelin)FABP. B-FABP is present mainly in astrocytes, H-FABP, the most prominent of all FABPs, in neurons and finally M-FABP is found in peripheral nerves (Pelsers and Glatz, 2005).

Their role could be demonstrated in all metabolic diseases, like diabetes, obesity and also in coronary artery disease, inflammatory conditions and in the CNS: in stroke, cerebral injury and neurodegenerative diseases, like Alzheimers and Creutzfeldt-Jakob disease. It is a fast-reacting injury marker, and after 2-3 hours B- & H-FABP rises in serum and stay elevated up to 120 hours. The cut-off serum concentration for cell injury is 5 - 6 $\mu\text{g/L}$. (Pelsers *et al.*, 2004)

8. Neuron-Specific Enolase (NSE)

NSE was first discovered in 1965 (Moore and McGregor, 1965) as an intracellular protein from the brain. Later it was described as a 78 kDa large, dimeric isoenzyme to a glycolytic enzyme, enolase. NSE can be

found in the neuron's cytoplasm; where it circulates with axoplasmic transport. (Cheng *et al.*, 2014) but normally it is not secreted out of the cell. It can appear in CSF and thereafter in the serum with neuronal damage. It is hardly detectable in healthy individuals but increasing rapidly with neural disruption. This quality makes it theoretically an ideal neuronal injury marker, explaining why it has been investigated intensively. Gradisek recommended it as prognostic biomarker and therapeutic indicator after traumatic brain injury. (Gradisek *et al.*, 2012; Olivecrona and Koskinen, 2012)

Recent meta-analysis in 16 studies found a strong correlation between NSE serum levels and outcome or death. (Cheng *et al.*, 2014) They found 100 % sensitivity for poor outcome and mortality at the levels of 11.6 – 20 $\mu\text{g/L}$ and > 20 $\mu\text{g/L}$ respectively. NSE rises within 12 hours of the neural injury and decreases within hours if the neural cell-disintegration ceases as the protein has a half-life of ~ 24 hours.

9. D-dimer (DDMR)

DDMR is fibrin degradation protein (FDP), which is a marker of the coagulation system activation. The coagulation system can be activated by intrinsic (endothelial activation) or extrinsic (tissue activating factors via Factor VII) pathways and both of them interconnect to fibrinogen, which in the presence of Ca²⁺ ions as cofactors and an activated thrombin converts to fibrinogen -

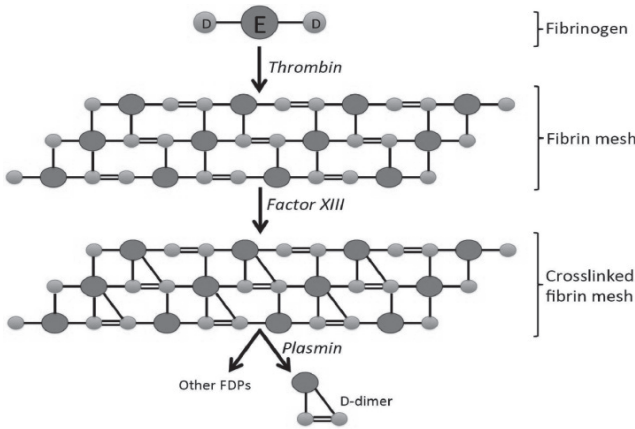


Figure 13. Coagulation mechanism and the early fibrinolysis, producing D-dimer (Public domain)

polymers and further with the help of a thrombin-activated Factor XIII to fibrin clot. The activated Factor XIIIa binds a glutamyl - lysyl amid crosslink which stabilises the blood clot. When the wound heals the coagulation stimulus ceases and fibrinolytic

mechanisms start to take over. The enzyme plasmin starts to break down the fibrin-clot to high molecular-weight polymers and then further to small polymers, FDPs. One of these FDPs is DDMR.

It received its name that fibrinogen's two D domains and one E domain is cross-linked together (Fig.13). It is normally undetectable in serum and increases rapidly when the coagulo-fibrinolytic system is activated, like

in DIC, thrombosis, DVT, PE, and sinus thrombosis. DDMR was discovered in 1973 and it came to routine use at the end of 1990s. It is tested with monoclonal antibodies with a reference value below 0.5 mg/L.

As SAH produces blood-clot and microvascular coagulation is suggested as causative mechanism for DCI, the coagulation system is involved in the pathological mechanism of the disease. This makes DDMR a neuro-marker to consecutively analyse.

AIMS

In this clinical study on patients with aneurysmal subarachnoid haemorrhage (aSAH) we aimed to investigate the following questions:

- ❖ To study the development of a well-known inflammatory marker C-reactive protein during the early course of the disease and investigate, if these changes can be associated to an established complication, cerebral vasospasm and the long-term outcome.
- ❖ To evaluate if a disadvantageous genetic variation in coding Apolipoprotein E has an effect on disease incidence, complication frequency and outcome after aSAH
- ❖ To elucidate, with the tools of genetics if proposed chromosome region 9p21 contains information to predispose for aSAH.
- ❖ To evaluate a novel investigation method, biochip-array neuropanel's applicability in aSAH patient-monitoring and its potential for outcome prediction.

PATIENTS AND METHODS

I. Inclusion

The study protocol was approved by the University Ethics Committee Gothenburg, Sweden with the allocation number S 161-00 and all the studies conformed to the Helsinki Declaration on human research.

All patients were admitted to the Neurointensive Care Unit at Sahlgrenska University Hospital, Gothenburg, Sweden between October 2000 and December 2002. They were consecutively considered for inclusion in the study if satisfying the following criteria:

- i. SAH was seen on CT-scan or detected with cerebrospinal fluid (CSF) analysis with increased red-blood-cell count or the detection of xanthochromia on CSF spectrophotometry.
- ii. The debut of symptoms, typically thunderclap headache with altered consciousness must have occurred within 48 hours of admission, although some patients might have experienced headaches previously (warning headache).
- iii. After admission, a cerebral angiography was performed with CT angiography or conventional digital subtractions angiography (DSA) with

3D image reconstruction and the intracranial aneurysm/s had to be identified in relation to the bleeding.

- iv. The patient had to be permanently residing in Sweden for long-term outcome follow-up.
- v. Finally an informed consent from the patient or next of kin had to be obtained within the first week of treatment.

202 patients fulfilled the inclusion criteria and were eligible for the studies but for other criteria listed beneath different numbers of patients were enlisted in the different studies.

II. Regime

Already after the diagnosis was established a first-physician neurological assessment was performed, (RLS, GCS, H&H, WFNS), previous and present medical conditions were noted, iv. tranexamic acid (Cyclocapron®) administered, and intensified monitoring was instituted. This involved invasive arterial pressure recording and systolic blood pressure management, keeping it below 160 mmHg even during transport from primary or secondary health care centres. Securing the airways and ventilation were mandatory if the patient became unconscious. After admittance to the university hospital, the patients were treated and observed in the intensive care unit, most of them in a specialised neuro-intensive care unit (NICU).

Patients were treated according to a standardised protocol which entailed securing the aneurysm by neurosurgical intervention (clipping of the aneurysm) or neuroradiological endovascular coiling after discussion between neurosurgeon and interventional radiologist. In some instances, when both therapy options were equally appropriate the patients were included in the International Subarachnoid Aneurysm Trial (ISAT), an international multicentre randomised study, where Sahlgrenska University Hospital participated between 1997 and 2002. (Molyneux *et al.*, 2002) In a few occasions (3 patients), conservative treatment with basic intensive care management was the therapy of choice. During the patient care the physiological and laboratory parameters were kept between strictly monitored limits, detailed in the

Introduction.

To reduce cerebral vasospasm (CVS), an early infusion of nimodipine (Nimotop[®]), a selective calcium antagonist were administered 2 mg/h for 10 days. In some mild cases, the patients received oral nimodipine (60 mg every 4 h) in the last 3 days. CVS was monitored with trans-cranial Doppler ultrasound (TCD) 3 times weekly or daily if spasm was discovered. CVS was registered if the peak systolic blood-flow velocity increased above 2 m/sec in the middle cerebral artery (MCA) or above 1.7 m/sec and concomitant neurological deterioration. The same applied if CVS was seen on CT or conventional angiography (DSA). These signs needed to lead to therapeutical measures *i.e.* increased nimodipine administration, volume therapy to counteract spasm or blood pressure

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 6	Day 8	Day 12-14	1 year
aSAH	√								
Inclusion	√	(√)	(√)						
Hunt&Hess	√	(√)	(√)						
WFNS									
ApoE/9p21	√	(√)	(√)						
Biochemical markers	√	√	√	√	√	√	√	√	
CVS		√	√	√	√	√	√	√	
Outcome									√

Table 9 Overview of the study plan. (aSAH - day of haemorrhage, marking means activity)

management.

During the patient treatment venous blood samples were collected on days 0, 1, 2, 3, 4, 6, 8, and between days 10-14. Physiological and laboratory parameters were registered and any surgical interventions recorded.

After one year (11-13 month), a complete neurological evaluation was performed and patient status recorded with GOSE, NIHSS and Barthel score by the same neurologist, blinded to the investigated (biochemical/genetical) markers. The study plan is summarised on Table 9.

III. Data collection and analysis

A. Paper I. (CRP)

202 patients were considered for inclusion with SAH, who were admitted to NICU. The following patients were excluded: 3 patients were admitted later than day 2 after the haemorrhage, in 33 patients, no aneurysm was detectable, no angiographic examination could be performed in 4 patients and no informed consent could be attained or the consent withdrawn in 9 cases. As described in the introduction craniectomy, as most major surgical interventions increase CRP, we excluded patients with surgical clipping as therapy options (39 patients) and have chosen to follow percutaneously treated

patients. As 3 patients were lost for follow-up and in 13 patients consecutive data were missing for AUC calculation, 98 patients remained in this patient selection. The patients' demographics and characteristics at admission and during observation can be seen in Table 1 in Paper I.

Data collection routines were described above (Table 1). Data analysis was performed instantaneously in the hospitals accredited laboratory with an immunochemical kinetic turbidimetry technique on a BH/Hitachi 917 analyser. (Roche-Diagnostic, 2001)

B. Paper II. (APOE)

196 consecutive SAH patients with *APOE* genetic information available, were considered to be included to this genetic study and 148 patients remained finally enrolled. Of those non-eligible (mostly the same as in Paper I), 3 patients were admitted later than day 2, no aneurysm was detected in 33 patients, no informed consent/consent withdrawal in 9 cases and finally 4 patients lost for long-term outcome. 105 patients treated with endovascular technique, in 39 patients the aneurysm was secured with neurosurgical clipping. 4 patients were conservatively treated since their clinical condition did not permit active intervention.

221 population-based, healthy individuals acted as control cohort, since we had the

intention to study the variant allele's effect on the incidence of aSAH. These healthy individuals were recruited from controls to other studies from the same geographical area (Blennow *et al.*, 2000; Prince *et al.*, 2004).

We collected blood at admission from the patients for genetic examination and at samplings' occasion from the controls (Table 1, Paper II). Genomic DNA was extracted from the samples with Geno-Prep kit (Genolution Pharmaceuticals Inc. Seoul, Korea) and GenoM-48 DNA purification system (GenoVision VmbH, Vienna, Austria), which is a magnetic particle-based technology. The solid phase for capturing and purifying nucleic acid (NA) uses magnetic stand, magnetic bead and chaotropic agents (GTC GuHCl) as lysis and binding agents, alcohols as washing and water as elution of isolated NAs. (Genolution Pharmaceutical, 2015; Blennow *et al.*, 2000). Genotypes were obtained using a solid-phase minisequencing method as previously described (Blennow *et al.*, 2000).

C. Paper III (9p21)

183 patients with verified aSAH who were admitted to the University hospital were enrolled in this genetical study where the analysis material was blood-sample drawn from the patients on the admission day. Their allelic frequencies were compared to 366

healthy controls, who were recruited from population based health survey (Wilhelmsen *et al.*, 1997) and the Swedish Population Register. These controls were matched for age, sex and geographical area. As recognised risk factors for SAH, hypertension and current or previous smoking habits were noted. As described in the Introduction the region 21 on chromosome 9p is recognised to show an association with intracranial aneurysms (IA) in a large GWAS on different populations (Bilguvar *et al.*, 2008) and also in a candidate gene study (Helgadottir *et al.*, 2008). Additionally more cardiovascular diseases were associated to this region like aortic aneurysm, coronary artery disease, arterial stiffness and also ischemic stroke. As different vascular properties are connected to this area (Wellcome Trust Case Control, 2007) it became interesting for susceptibility for intracranial vascular weakness, thereby aneurysm formation.

A 44 kbp (kilo base-pair) region was tagged by using HapMap Central European (CEU) genetical data and Haploview 4.1 program (Broad Institute; Cambridge, MA, USA). This tagging with $r^2=0.8$ and minor allele frequency (MAF) of 0.1, resulted in six single-nucleotide polymorphisms (SNPs): rs10965227, rs1547705, rs7857345, rs1333045, rs1333040, and rs10757278. To further analyses, we added one extra SNP,

rs1537378 recently found in association with ischemic stroke and large vessel disease although it was not in the same (linkage disequilibrium) LD structure with the others. We used TaqMan Custom Assays to genotype patients and controls with primers and probes specially made at Applied Biosystems (Carlsbad, CA, USA). 384 Well GeneAmp PCR system 9700 was used for amplification and fluorescence imaging was carried out on an ABI PRISM 7900HT Sequence Detector (both from Applied Biosystems). The analyses were blinded to patient-control status. One assay, rs1333040 had technical problems and could not be repeated. The 5 successfully genotyped SNPs tagged 90% of the SNPs in HapMap in the 9p21's region of interest.

D. Paper IV (Neuropanel)

The aim of this study was to evaluate a novel neuromarker biochip array and to test if the course of the 9 markers in the array can be associated to initial neurological condition measured with Hunt and Hess scale (H&H), cerebral vasospasm (CVS) during the first two weeks or long-term general outcome measured with the Extended Glasgow Outcome Scale (GOSE), the focal neurological deficit with the National Institute of Health Stroke Scale (NIHSS) and their activity of daily living with the Barthel Index score. We wanted to assess if the markers individually or together could

predict any of these parameters during the course of aSAH.

We aimed to select randomly approximately 40 patients equally distributed among the H&H categories (H&H 1-5) with full set of blood samples (see above). 41 patients were recruited and their demographics, disease, therapy, complication and outcome attributes are presented in Table 10.

In the next subchapter I describe the clinical scales utilised and how we dichotomise them for easier clinical interpretation.

We tested the selected series of serum samples for the putative brain damage markers' concentration (GFAP, IL6, CRP, NGAL, NSE, BDNF and DDMR) in a biochip array cerebral panel (Randox, Crumlin, UK). It is a semi-automated system, where the array is a solid substrate, containing the immobilised specific antibodies in separate areas for separate protein markers. The technique is designed to simultaneously quantify the different substrates in a drop (35-100 μ l) of serum, plasma or cerebrospinal fluid. The analyses were performed blinded for the clinical data.

Hunt & Hess	All patients	Sex	Age	CVS	Op/Embol	
categories	No. (%)	Male/Female	(Mean, Min, Max, SD)	Spasm / No spasm	Surgical / Radiol.	
H&H 1	8 (19.5%)	5 / 3	51.3 (37, 66, 12.5)	3 / 5	1 / 7	
H&H 2	9 (22.0%)	0 / 9	48.2 (20, 81, 21.7)	4 / 5	1 / 8	
H&H 3	9 (22.0%)	2 / 7	56.0 (42, 65, 8.5)	5 / 4	1 / 8	
H&H 4	10 (24.4%)	0 / 10	60.0 (40, 75, 8.9)	4 / 6	1 / 9	
H&H 5	5 (12.2%)	1 / 4	54.2 (39, 69, 10.8)	4 / 1	0 / 5	
Total	41 (100%)	8 / 33	54.2 (20, 81, 13.5)	20 / 21	4 / 37	
χ^2 (p-value)	-	0.007	0.575	0.575	0.958	
GOSE		Outcome	NIHSS		Barthel	
GOSE 1	7 (17.1%)		NIHSS 0 p	23 (56.1%)	Dead	7 (17.1%)
GOSE 2	0 (0.0%)		NIHSS 1-10 p	7 (17.1%)	0-9 p	4 (9.8%)
GOSE 3	6 (14.6%)		NIHSS 11-20 p	2 (4.9%)	10-39 p	1 (2.4%)
GOSE 4	0 (0.0%)	Poor 13(31.7%)	NIHSS 21-42 p	2 (4.9%)	40-99 p	2 (4.9%)
GOSE 5	8 (19.5%)		Dead	7 (17.1%)	100 p	27 (65.9%)
GOSE 6	8 (19.5%)		For categorical variables n (%) is presented.			
GOSE 7	5 (12.2%)					
GOSE 8	7 (17.1%)	Favorable 28(68.3%)				

IV. Clinical and radiological assessment

The different types of scales, admission and outcome assessment tools are detailed with explanation and background in the Introduction. I intend to give only a brief recapitulation which assessment measures were used in the different papers.

A. Paper I (CRP)

We used WFNS, Fisher scale as admission assessment, CVS, infection status during the observation and outcome measures after one-year with GOSE, NIHSS and Barthel score. GOSE was dichotomised in prognostic statistical calculations to poor (GOSE 1-4)

Table 10. The demographics, clinical and outcome parameters of 41 patients in Paper IV. (CVS-cerebral vasospasm, Op/Embol- therapy modality, outcome parameters (GOSE, NIHSS, Barthel and how we dichotomised the scales)

and favourable (GOSE 5-8), NIHSS, as no focal deficit detected (NIHSS=0), and patient with focal deficit (NIHSS>0). Finally the Barthel Index was dichotomised as no help required during daily ADL (Barthel=100) and assistance needed (Barthel<100).

B. Paper II (APOE)

We utilised Hunt and Hess scale as admission neurological assessment, CVS was measured and followed and one-year

	Paper I	Paper II	Paper III	Paper IV
Kolmogorov-Smirnov test	√			
Spearman's rank correlation test	√	√		√
Wilcoxon's signed rank test	√			
Univariable logistic regression	√	√	√	√
Multivariable logistic regression	√		√	√
ROC	√			√
LR, PPV	√			
Sample size calculation	√	√		
Pearson's χ^2		√		√
Mantel-Haenszel test		√		
Hardy-Weinberg equilibrium test		√	√	

Table 11 The applied statistical methods for the individual papers. Abbreviations: ROC, -receiver operator characteristic curve, LR, -likelihood ratio, PPV, -positive predictive value

outcome evaluation was performed with GOSE, NIHSS, and Barthel score. Dichotomisation was performed as in Paper I. Area and gender matched control group with known *APOE* constitution was recruited.

C. Paper III (9p21)

Only incidence of aSAH was confirmed and genetical variation investigated in a case-control study.

D. Paper IV (Neuropanel)

Hunt and Hess scale was utilised as selection criteria and the levels of 9 biochemical markers were followed and the course was compared to H&H status, the presence or absence of CVS and outcome parameters with GOSE, NIHSS, and Barthel score.

V. Statistics

Statistical analyses were performed with SPSS 21.1 (Statistical Packages Software System – IBM Corp. Armonk, NY, USA) and SAS 9.4 (Statistical Analysis Software – SAS Inst. Inc. Cary, NC, USA). In planning (*i.e.* sample size calculation) and result interpretation (correct statistics) professional statistical expertise was entrusted (Statistiska Konsultgruppen, Gothenburg, Sweden). I listed the statistical methods, employed in the separate papers in the table below. (Tabl. 11)

Apart from the above mentioned statistical methods, one additional genetic statistical software was utilised in Paper III in conjunction with haplotype frequency calculation. The THESIAS[®] Java based program is constructed to performed

haplotype-based association analysis in to a reference haplotype (Tregouet and
unrelated individuals. This program is based Garelle, 2007).
on the maximum likelihood model and
estimates ORs for each haplotype in relation

RESULTS

I. Biochemical neuromarkers (BNMs)

A. Neuron cell-injury markers:

The BNMs below comprise an admixture of proteins recognized to be found in the central nervous system. They have been shown to have variable functions like structural-, transport-, receptor- and special purpose proteins within inflammation and coagulation. The common attribute of these proteins is that all of their concentrations in body fluids have been found to increase after neuronal injury. We have investigated

nine BNMs in a pilot study in 41 patients after aSAH in Paper IV. The correlation calculations between the investigated BNMs and admission neurology, CVS and outcome measurements (GOSE, NIHSS, Barthel) are described in Table 12.

Neuron Specific Enolase (NSE)

NSE followed an irregular pattern, but a continuous rise from Day 0 (mean 2.82 ng/ml, SD 2.78) to Day 12-14 (8.57 ng/ml, SD 7.38) could be observed. The maximum or mean values showed, however no correlation with H&H, vasospasm or the

Spearman Correlation Coefficients, N = 41					
Prob > r under H0: Rho=0					
	Hunt &Hess	CVS	GOSE	NIHSS	Barthel
BDNF_mean	-0.22669	0.00412	-0.02352	0.10207	-0.15120
	0.1541	0.9796	0.8840	0.5254	0.3453
IL6_mean	0.36435	0.51137	-0.46370	0.32305	-0.40355
	0.0192	0.0006	0.0023	0.0394	0.0089
FABP_mean	0.46388	0.52374	-0.59631	0.57745	-0.53179
	0.0023	0.0004	<.0001	<.0001	0.0003
GFAP_mean	0.05803	-0.05193	-0.11849	0.09976	-0.06626
	0.7186	0.7471	0.4606	0.5349	0.6806
CRP_mean	0.45780	0.33405	-0.32876	0.30564	-0.29538
	0.0026	0.0328	0.0358	0.0520	0.0608
DDMER_mean	0.76738	0.31342	-0.42117	0.35675	-0.37604
	<.0001	0.0460	0.0061	0.0220	0.0154
NSE_mean	0.02228	0.27218	-0.14251	0.05171	-0.14376
	0.8900	0.0851	0.3741	0.7482	0.3699
NGAL_mean	0.14462	0.33404	-0.27972	0.38044	-0.32185
	0.3670	0.0328	0.0765	0.0141	0.0402
TNFRI_mean	0.23337	0.32167	-0.48651	0.50398	-0.55206
	0.1420	0.0403	0.0013	0.0008	0.0002

Tabell 12 Correlation table for neuropanel markers; max and mean values vs. Hunt &Hess scale, CVS, GOSE, NIHSS and Barthel index. Spearman's rho coefficients and p-values are presented. Statistically significant values are highlighted and marked in grey.

outcome parameters (GOSE, NIHSS, Barthel).

Brain-Derived Neutrophic Factor (BDNF)

BDNF did not follow any specific pattern during the observation period in our patient group. The mean values stayed around 1000 pg/ml with daily variations ± 100 pg/ml. Consequently, no correlation could be discovered with any of the study parameters.

Fatty Acid Binding Protein (FABP)

FABP, a group of lipid-transport proteins, first derived from central and peripheral nervous system tissues, were also included in our investigation. It appeared to be a fast reacting marker with the highest values on day 0 (mean 6.67 ng/ml, SD 5.01) and continuously falling to day 3 (2.69 ng/ml, SD 2.18), thereafter increasing again, reaching a second peak on day 8 (4.56 ng/ml, SD 7.45). FABP correlated strongly, in contrast with the above markers, to all study parameters. (Spearman's Rho 0.39-0.57), $p=0.022$ - <0.0001). The univariable ORs ranged between 1.43-2.19 and p values

0.025-0.004. FABP had the best predictive qualities for CVS with $ROC_{AUC}=0.80$ of all the measured BNMs. Besides, FABP proved to be a good predictor even for the outcome parameters (GOSE: $ROC_{AUC}=0.85$, $p=0.019$; NIHSS: $ROC_{AUC}=0.83$, $p=0.004$; Barthel: $ROC_{AUC}=0.83$, $p=0.025$). Figures 14, 15, 16, 17, 18, & 19 present the temporal course of those BNMs mentioned above and their mean values are depicted with standard error of the mean (SEM) split between the dichotomous groups of the parameters investigated. The unfavourable group, painted grey, represent the number of patients with CVS, $n=20$ (48.8%), poor outcome with dichotomised GOSE, $n=13$ (31.7%), neurological deficit with NIHSS, $n=18$ (43.9%) and finally number of patients in need of help with ADL, Barthel $n=14$ (34.1%). All tests were performed with univariable logistic regression. P -values, ORs and Area under ROC curves (ROC_{AUC}) are based on original values not on the stratified groups. OR is the ratio for the odds for an increase of the predictor with one unit.

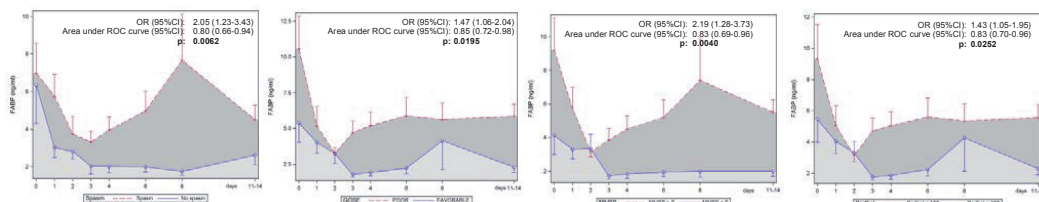


Figure 14. The course of Fatty Acid Binding Protein (FABP)

B. Glia cell-marker

Glial Fibrillary Acidic Protein (GFAP)

This glia cell-injury marker has been investigated in a previous study in partially overlapping aSAB patient material (Nylen et al. 2007). In our 41-patient material with the new biochip-array technique, we could not find an association between either the maximum or mean values of GFAP and the initial neurology (H&H), cerebral vasospasm (CVS) or any of the outcome

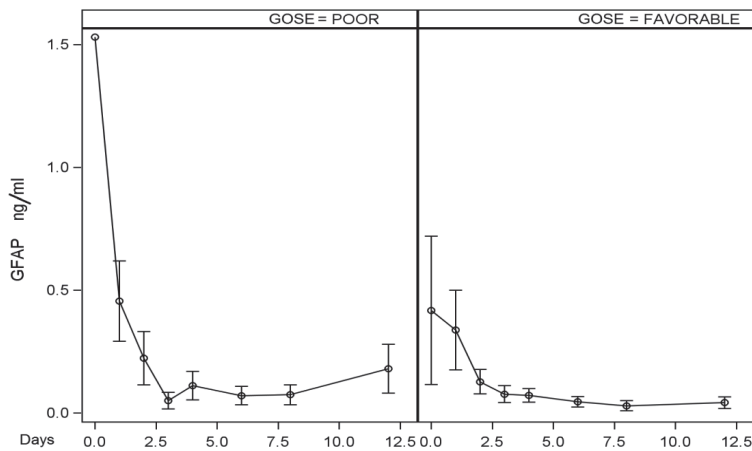


Figure 15 The course of GFAP in patients with poor and favourable outcome (dichotomous GOSE). Mean values and standard error of the mean (SEM) are presented

parameters tested. (Spearman's Rho was at best $Rho = -0.19$, $p=0.22$). However, when plotting the results, there was a tendency of the poor outcome patients having higher initial values (Fig.15).

C. Coagulation protein

D-dimer (DDMR)

DDMR is a fibrin degradation protein which is a product of fibrinolysis, a natural process of the organism dissolving a thrombus or a clot. As SAH entails a clot-formation, it has been proposed that the level of DDMR corresponds to the amount of blood exsanguinated from the aneurysm and consequently to the risk of vasospasm and outcome (Fujii et al. 1997). As DDMR is included in the neuropanel biochip of

Randox® UK, we aimed to test this coagulation protein, relatively new to SAB diagnostics.

We found initially medium high values (mean: 376 ng/ml, SD: 503) which decreased

gradually, reaching its nadir

on day 2 (180 ng/ml, SD: 134), thereafter increasing again to even higher levels and staying high during the whole observation period (day 12-14: mean 477 ng/ml, SD: 372). Although, there is no difference in the initial course and levels of DDMR between the favourable and unfavourable group, the

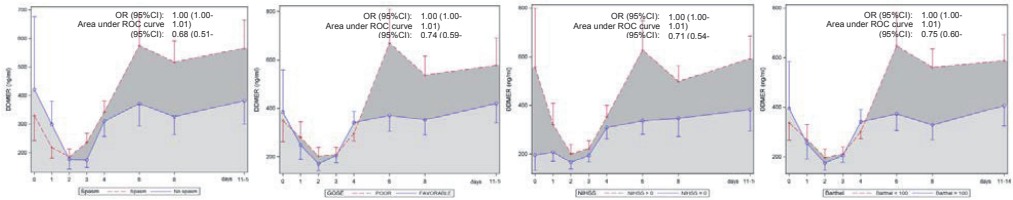


Figure 16. The course of DDMR in patients in the dichotomous observation parameters (CVS, GOSE, NIHSS, Barthel)

gap becomes noticeable in the late course (after day4) between these cohorts. The correlation is nevertheless significant between the course of DDMR and H&H ($Rho=0.77$, $p<0.001$), CVS ($Rho =0.31$, $p=0.046$) and all of the outcome parameters. Even though mean values give significant correlations to all outcome measures, interestingly the maximum values give 25-30% higher correlation coefficients, hence considerably lower p values. This characteristic is unique among BMNs. Because of the similar patterns between patients with poor and good outcome up until day₃₋₄, the DDMRs predictive value reaches significance only to calculate focal deficit (NIHSS; $ROC_{AUC} = 0.71$, $p=0.041$). (Fig.16)

D. Inflammation markers

Probably the most prominent reaction occurring after the “early injury” of the initial haemorrhage is inflammation. It is the

tissue’s reaction to foreign substances, and part of the immunological processes of healing. It is one of the most studied pathological systems and hundreds of mediators, substances, ligands and receptors are described to be involved in this process. Randox[®], UK-s immunological biochip-plate has chosen four of them to be included in their diagnostic neuro-panel.

Neutrophil Gelatinase-Associated Lipocalin (NGAL)

NGAL, or Lipocalin-2 has only been previously investigated in one study in patients with aSAH (Serra et al. 2014). NGAL showed a weak, nevertheless significant correlation to CVS ($Rho=0.33$, $p=0.032$), NIHSS ($Rho=0.38$, $p=0.014$) and Barthel ($Rho=-0.32$, $p=0.040$). The temporal course of the marker showed a clear difference between the worse/better patients in regard of CVS, GOSE, NIHSS, and

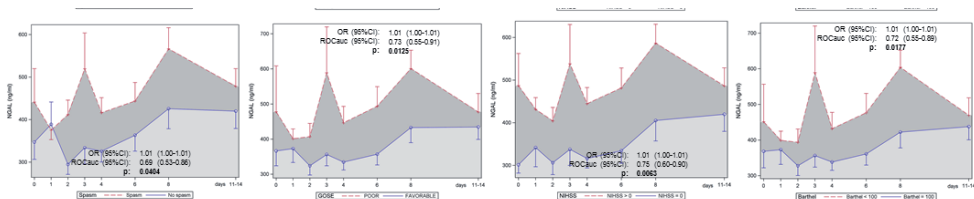


Figure 17 The course of NGAL between the worse and better patients with regards of the scales measured

Barthel. The predictive qualities were all significant, ranging between $ROC_{AUC}=0.69-0.75$. (Fig. 17)

Tumour Necrosis Factor Receptor 1. (TNFR1)

TNFR1 is one of the receptors of $TNF-\alpha$, which is recognised to act as mediator in inflammatory processes. The soluble TNFR1 and 2 are activated by $TNF-\alpha$ and having a longer half-life than the actual cytokine, thereby acting as a more reliable measure of inflammation activity than its ligand. TNFR1 demonstrated a continuously

conclusion: excellent predictor according to Swets (Swets 1988). (Fig.18)

Interleukin-6 (IL-6)

IL6 is one of the most important pro-inflammatory cytokines and mediator of fever through prostaglandin (especially PGE_2) production. As found in smooth muscle cells in blood vessel walls it is involved in vessel regulation during inflammation. It has recently been assigned new functions as neuropoietin for its positive neural properties (Vezzani and Viviani 2015). Not surprisingly, IL6 is

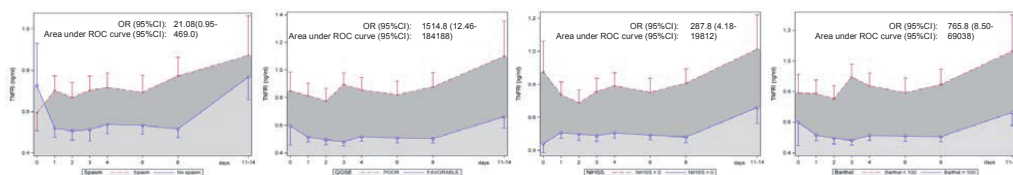


Figure 18. TNFR1's course with CVS and outcome measures

rising pattern. The correlation between the early diagnostic scales are weak (H&H – non significant, $CVS - Rho=0.32, p=0.040$), but becomes strong ($Rho>0.5, p<0.01$) with the outcome measures. It has the best predictive strength of all the BNMs investigated for poor – good outcome with $ROC_{AUC}=0.87, p=0.0028$, bordering the

interesting as a marker of inflammation and regeneration after SAB. As nearly all cytokines are fast reacting mediators, IL-6 demonstrated rapid rise after the insult, reaching its peak on day2-4. The correlation was good on all the observation parameters ($Rho=0.36-0.51, p=0.019-0.0006$), but due to its fast reaction and with regard of its

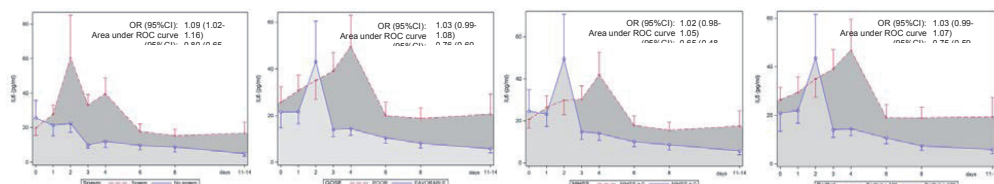


Figure 19. The development of Interleukin-6 (IL6) during the first two weeks in patients with or without vasospasm and favourable/unfavourable dichotomous groups of outcome scales

peak, it showed predictive quality only with CVS (ROC_{AUC}=0.8, p=0.0097). (Fig.19)

remaining at a higher level in the unfavourable group. Although the values

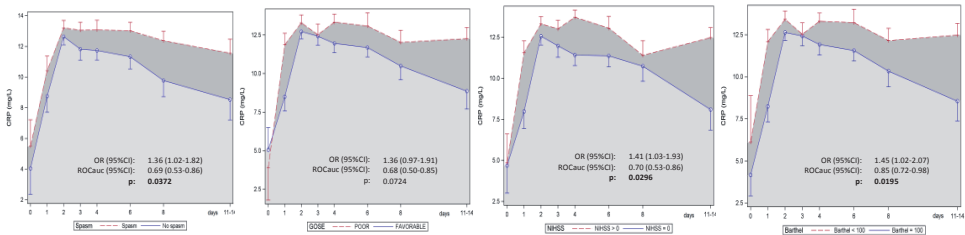


Figure 20 HsCRP-s progress after aSAH plotted separately for favourable/unfavourable patient-groups

1. C - Reactive Protein (CRP)

CRP measurement, as the most accepted and utilised inflammation marker, was included in the Randox[®] UK-s stroke-diagnostic neuro-panel. As this biochip-controlled test was originally designed to diagnose ischemic stroke, it included the high-sensitivity CRP (hsCRP) assay version with a dynamic range of 0-15 mg/L. We found that hsCRP reached the upper limit soon after the bleeding in many cases, and stayed there 3-4 days. It decreased rapidly without normalising in the favourable patient-group and more slowly,

struck the ceiling of measurement in several cases, it showed nonetheless significant correlation to most of the clinical rating scales (H&H, CVS, GOSE, Barthel).

CRP's prospective value as a neuromarker (BNM) was explored comprehensively in Paper I and its potential as an outcome predictor was evaluated early in the course of aSAB. 98 consecutive patients with

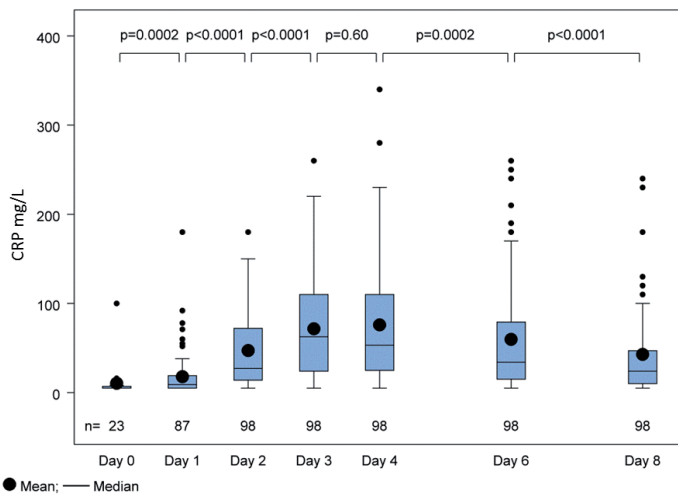
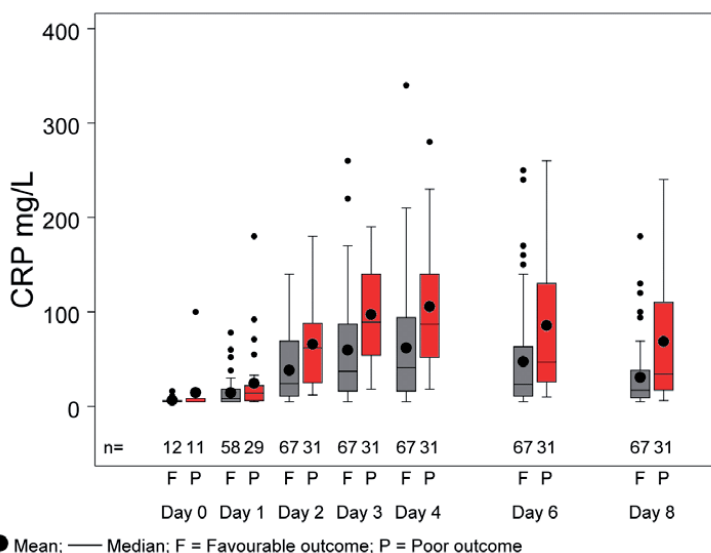


Figure 21 Box plot of CRP values from day 0 to day 8 with median, mean and IQR. Differences tested with Wilcoxon's signed rank test.

endovascularly treated aneurysms were enrolled in this study. The patients' demographics, neurological status at first examination, radiological grading, infectious status during observation and their CRP values during the first week of treatment are described in Table 1 in Paper I. We could demonstrate a continuous rise of CRP values from normal at day 0 (median 5 mg/L, IQR 5-7) to its peak at day 3-4, (median value 53 mg/L, IQR 24-100). The values decreased after this peak until day 8 without normalising. (median 24 mg/L, IQR 10-47). The changes between each subsequent day were significant, apart from the peak. The course of CRP levels in the first 8 days is depicted in Fig.21.



● Mean; — Median; F = Favourable outcome; P = Poor outcome
 Figure 22 Box plot of CRP values from day 0 to day 8 with median, mean and IQR. Differences tested with Wilcoxon's signed rank test

This change was independent from the status of infection. We were the first to describe this temporal course (Csajbok et al. 2005) and were intrigued by the result, which corresponded with our clinical experience. We tested if there was a difference in the course of CRP development between the patients with unfavourable and favourable outcome. The same boxplot, split between these patient groups confirmed the result (Fig.22).

The linear regression lines showed a stronger association between the mean CRP values in the first week and long-term outcome, measured by GOSE, compared to the radiological scale Fisher or initial neurology categorised by the World

Federation of Neurological Surgeons Scale (WFNS) in this patient group. (Fig.23) The correlation coefficients (Spearman's Rho) confirmed the stronger connection between CRP_{mean} and CRP_{max} to GOSE (-0.49, -0.45, p<0.0001) and NIHSS (0.45, 0.45,

p<0.0001) compared to Fisher scale (-0.25, p=0.01; 0.16, p=0.16) and WFNS (-0.44, p<0.0001; 0.27, p=0.001).

with p-values. The receiver operator curve (ROC) was drawn to estimate the parameters' predictive strength and choosing

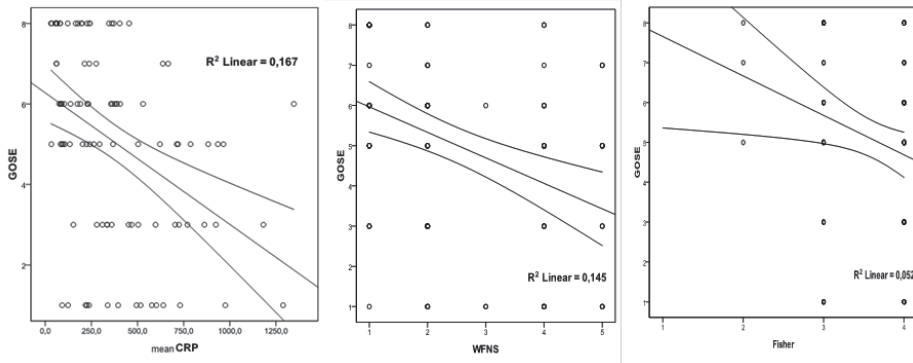


Figure 23 GOSE – CRP_{mean}, GOSE – WFNS score and GOSE-Fisher scatter plot with linear regression line and 95% confidence interval of the mean

As CRP's correlation to outcome was comparable or better than initial clinical status or radiological evaluation, we tested its predictive qualities in a univariable logistic regression model. In Table 13, the univariable odds ratios (OR) were presented

a cut-off point. (Fig. 24)

In a multivariable logistic model, after adjustment for age, sex, WFNS and Fisher scale, the CRP values were the only variable which remained significant in relationship to

Variable	OR (95%CI)	p-value	Area under ROC-Curve (95%CI)
Gender	1.42 (0.54-3.72)	0.48	0.53 (0.44-0.63)
Age (years)	1.01 (0.97-1.05)	0.58	0.54 (0.41-0.66)
Neurological status at first attention (WFNS score)	1.44 (1.06-1.94)	0.019	0.67 (0.56-0.77)
Radiological classification, Fisher score	1.91 (0.80-4.57)	0.14	0.57 (0.47-0.67)
Infectious status during observation	1.81 (0.74-4.42)	0.19	0.57 (0.47-0.67)
CRP max (OR per 10 units)	1.12 (1.05-1.19)	0.0009	0.74 (0.64-0.84)
CRP mean (OR per 10 units)	1.25 (1.10-1.42)	0.0006	0.76 (0.66-0.86)
CRP day 1 (OR per 10 units)	1.19 (0.96-1.48)	0.1115	0.62 (0.48-0.73)
CRP day 2 (OR per 10 units)	1.18 (1.06-1.32)	0.0030	0.70 (0.60-0.80)
CRP day 3 (OR per 10 units)	1.13 (1.04-1.22)	0.0037	0.72 (0.62-0.82)
CRP day 4 (OR per 10 units)	1.11 (1.03-1.19)	0.0048	0.73 (0.63-0.83)

Table 13 Univariable logistic regression analysis - prediction of poor outcome

outcome.

The area under the ROC curve (AUC_{ROC}) that can describe accuracy of the test (Swets 1988) showed moderate accuracy for CRP_{mean} and CRP_{max} (0.76, $p < 0.001$; 0.74, $p < 0.001$), poor accuracy for WFNS (0.67, $p = 0.008$) and no predictive value

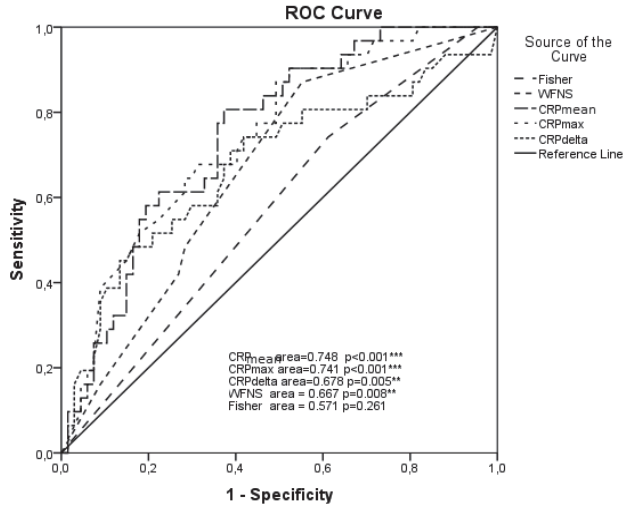


Figure 24 ROC curve of CRP_{mean} , CRP_{max} , CRP_{Δ} , WFNS score and Fisher scale for prediction of outcome

for Fisher scale (0.57, $p = 0.14$), rendering WFNS and Fisher scale inapplicable for outcome prediction. In our endeavour to find even earlier prognostics, we added CRP

values on day 2 and 3 to our ROC analysis and found surprisingly, that already CRP_{day2} provided better prognostic accuracy ($AUC_{ROC} = 0.7$, $p = 0.002$) than WFNS and Fisher scale.

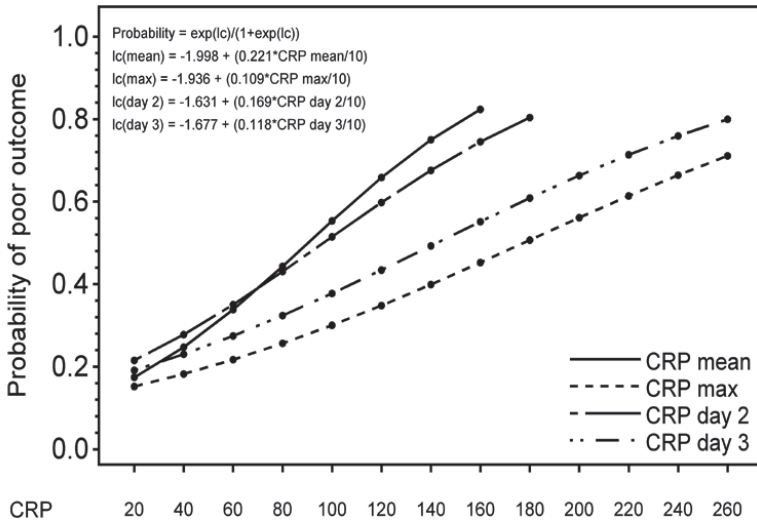


Figure 25 Probability of poor outcome expressed as a measurement of CRP on day 2 and day 3, CRP_{mean} and CRP_{max} during the first week after aSAH calculated from the logistic regression model

The logistic regression analysis could offer us a probability curve to predict poor outcome for the best predictors in relation to the CRP values (Fig.25). This is the first time a practical

probability estimation has been provided based on early CRP values.

II. Genetic neuromarkers (GNM)

A. *ApolipoproteinE* gene (*APOE*)

The *APOE* $\epsilon 2/\epsilon 3/\epsilon 4$ polymorphism is probably the most investigated genetic allele variation in connection with neurological diseases and pathophysiology. As described in the introduction two single nucleotide polymorphisms (SNPs) rs7412 and rs429358 on chromosome 19 are associated with three different allele variants: $\epsilon 2$, $\epsilon 3$ and $\epsilon 4$. As *APOE* $\epsilon 3$ is the wild type, and most common allele and $\epsilon 4$ is the variant with the strongest association with neurodegeneration (Alzheimer's disease in particular), we have studied the effect of *APOE* $\epsilon 4$ on the incidence, course, complications and outcome of aSAH in West-Swedish population and patient cohorts in Paper II. As described in Table 1 of Paper II, we had 148 patients, with aSAH and compared *APOE* genotype frequencies in this group to those in a control group consisting of 221 healthy individuals.

The result was surprisingly unambiguous despite previous studies' contradictory conclusions. The controls and patients were matching in all aspects (gender, geographical area) apart from age (patients mean=55.3 years, controls mean=72.3, $p < 0.001$). The different cohorts' *ApoE* constellation showed, however virtually the same $\epsilon 4$ proportions. ($p = 0.96$) As in other studies (Linn et al. 1996), gender proved to be a risk factor for aSAH, as 73% of the patients were female (108), compared to 27% male (40), resulting in a univariable OR of 1.86 (1.19-2.92, $p < 0.001$). No differences in allelic frequencies were noted between the sexes in controls ($p = 0.15$) and patient ($p = 0.79$). (Fig.26)

Among the therapy alternatives (surgical, endovascular and conservative), no difference was registered according to the patients' $\epsilon 4$ carrier status. ($p = 0.68$)

As one can clearly note from Fig.16, neither in vasospasm (CVS) nor in long-term outcome measures, assessed with GOSE, NIHSS, and Barthel score, the *APOE* $\epsilon 4$ carrier frequencies differed significantly.

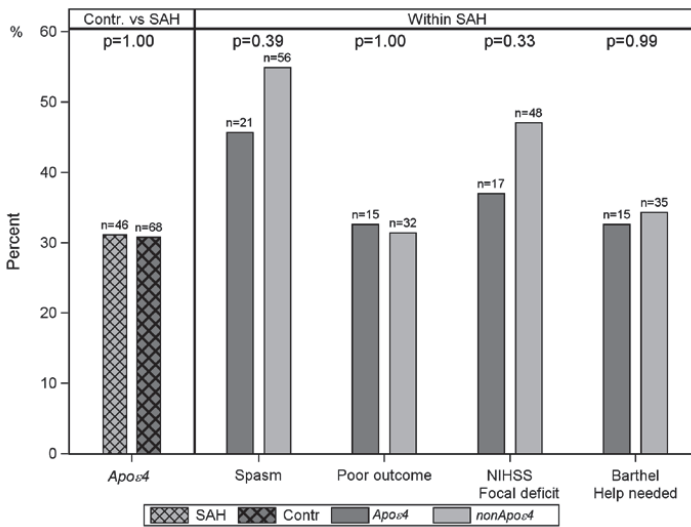


Figure 26 Proportion of APOE ε4 and nonAPOE ε4 allele carriers in healthy controls and SAH patients, in SAH patients with CVS and in SAH patient with poor outcome after one year. (SAH – subarachnoid haemorrhage, CVS – cerebral vasospasm (Spasm), Poor outcome – Glasgow Outcome Scale Extended (GOSE) 1-4, Focal deficit - National Institute of Health Stroke Scale (NIHSS) = 0 APOE ε4 – Apolipoprotein E gen, allele 4)

B. Chromosome 9p21

As we did not find an association between subarachnoid haemorrhage's pathophysiology and the well-known, previously described potential susceptibility gene on chromosome 19q13.2, we turned to the Department of Genetics at the Sahlgrenska University Hospital to identify other possible genetic markers.

In Paper III, we described a genetic study where variants of a gene in a particular chromosome-segment were investigated. We examined whether an association could be identified between single nucleotide polymorphisms (SNPs) and subarachnoid

haemorrhage by comparing the patients' and controls' genetic configuration at those particular base-pairs.

With genome wide association studies (GWAS) in European and Japanese populations chromosome 9p21 was linked to intracranial aneurysms (IA) (Bilguvar et al. 2008).

A 44 kbp (kilo base-pair) long DNA sequence was searched with the help of the HapMap database and the HaploView program. This exercise resulted in six tagged SNPs (Fig.27).

The genotype frequencies at those base-pairs were analysed both in patients and controls and the uncommon allele determined. One SNP, rs10757278 emerged already at this stage as the only SNP showing significant difference between controls and patients. (p=0.02) The uncommon alleles were subsequently inserted in a univariable regression analysis and ORs were calculated. Two of the alleles showed significant ORs to develop aSAH.

As smoking and hypertension were identified as main modifying factors, we determined if the SNPs showing association with aSAH were independent from these recognised risk elements. Multivariable regression model showed significance solely for the same SNP, rs10757278 G allele, as an independent marker. It showed even additive effect for homozygous GG subjects with univariable ORs of 1.38 (1.18-1.63) and 1.72 (1.39-2.13) for heterozygous and homozygous individuals respectively.

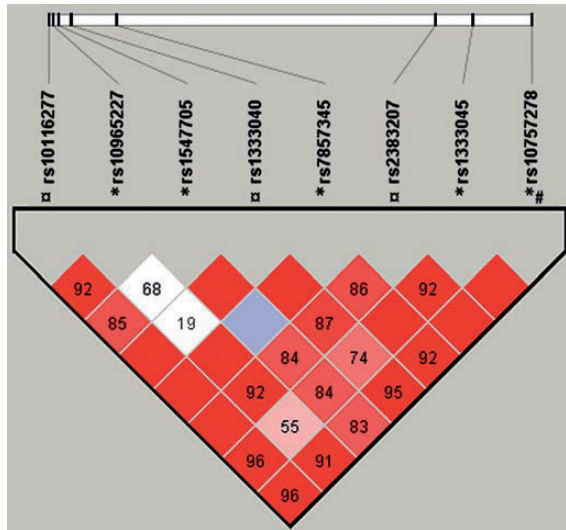


Figure 27 Graphic representation of the linkage disequilibrium (LD) structure in the region 9p21, downloaded from the HapMap database, showing SNPs, analysed in this and other studies. (* present study, □ Bilguvar et al, # Helgadottir et al)

DISCUSSION

I. General considerations

In our studies, we have focused on a patient - group with aneurysmal subarachnoid haemorrhage. We have explored different biochemical and genetic markers' relation to the incidence of the bleeding, the course during the disease development, the complication frequency and the different measures of outcome (Papers I-III). In the final study, we evaluated the usefulness of an innovative biochip-array for monitoring and prediction of outcome in this patient cohort (Paper IV). Our main findings were the associations or the absence of associations, between these neuromarkers and one or more aspects of this devastating haemorrhagic stroke entity. Some of the links we have discovered were strong enough to enable us to make predictions about general outcome. Our overall aim was to look at these results from an intensive care physician's point of view but with a neurologist's sense for details and a neurosurgeon's focus on the whole picture. Our tools, to achieve this were a prospective cohort patient population with a complete inclusion, to gather so many patients as possible, daily or almost daily serum sampling, noting all details of the

condition and treatment of the patients and finally a meticulous long-term follow-up.

We lost a mere 0.6 % (12) of the identified, eligible patients from inclusion during the two-year study period, which stretched over three years from inclusion to final outcome assessment. The study borders feasibility for a single-centre in this respect. Retrospective, multi-centre or meta-analysis studies are the next step to increase the number of participants with other types of methodological problems to consider. We had a general unfavourable outcome, *i.e.* death or dependency of 32% among the aSAH patients which is in line with other large studies (the ISAT study) (Molyneux *et al.*, 2002; Rosen and Macdonald, 2004) and even the proportion of poor grade subarachnoid haemorrhage was corresponding with 22% as in Rosen's study with 3567 participants. This indicates that our study-population has similar distribution as larger studies in the world, which makes our conclusions generally applicable for other subarachnoid patient groups in similar hospital environment.

These studies were not designed to compare therapy alternatives. The figures were merely described as methodological information for parallel interpretation with other studies in the literature. The majority, 71% of our patients were endovascularly treated; surgical

clipping was therapy alternative in 26% and 3% were treated conservatively *i.e.* basic intensive care. These figures vary both geographically and in time in the literature. In a Chinese centre all good-grade aSAH patients (H&H 1-2) were operated in 2003, the rest were conservatively treated (Tang *et al.*, 2003), while in a Scottish centre 11% of the patients operated and 89 treated with coiling (Teo *et al.*, 2015). At the same department, the therapy trend changed in favour of coiling from 11% in 1995 to 89% in 2003 and has continued on that level since then. This paradigm shift happened after the ISAT study, although the difference in RR (relative risk) reduction between the endovascular vs. neurosurgical treatment groups was only 15% with 2-3% higher risk of re-bleeding in the coiling group. The 18-year follow-up of this material has been published this year (Molyneux *et al.*, 2015) and it showed that the difference in dependency is not significant, but the chance for being alive 10 years after treatment is higher in the coil-group (OR: 1.34), however with 1,7% re-bleeding risk from the target aneurysm.

II. Patient considerations

There is one weakness in all third-level referral hospital driven clinical trials; they all have flawed inclusion, however thoroughly

they try to avoid it. There is an important group of patients in poor condition, who never reach these university hospitals, thereby the public impact of the diseases is much larger than the actual figures and the outcome is worse than claimed (Nylen, 2007). Unfortunately it is only disease demographic studies, based on records and data-bases which can put these figures right.

The positive fact is that different data exist to assess the initial status of SAH patients and the severity of the haemorrhage: clinical-, radiological-, laboratory examination, lumbar puncture and not the least the experience of the clinician. They complement one-another and finally they form a basis for decision with quite high sensitivity and specificity. To have the ambition that one sole modality, let alone a single marker would substitute this complex decision-making system is over-pretentious.

One of the more challenging facts is the diversity of clinical picture the subarachnoid haemorrhage patients present. There is a large dispersion in the time-frame they appear in the emergency department, from a few minutes to a couple of days or even weeks. There were some patients, presenting late on the medical or neurological wards with mild symptoms, which later could be related to aSAH. Most of these patients were unfortunately ineligible for our studies, elapsing more than 2 days from the bleeding.

Seizures present an extremely complicating dimension to initial assessment. Nearly one in ten patients was found having early-onset seizures (EOS) at admission (Fung *et al.*, 2015), of whom 66% were graded poor on clinical scales. 68% of these patients achieved nevertheless good outcome at 6 month. Furthermore, patients having seizures are treated on site with anti-epileptics (often benzodiazepines) which affect the level of consciousness, resulting in worse clinical scores than the bleeding would have warranted. This fact adds to the explanation why initial neurology fails to predict outcome reliably. (Paper I) These seizures may even proceed to non-convulsive seizures (NCSs) that have been implicated in the aetiology of secondary brain injury. Claassen and co-workers observed 479 SAH patients and found 11% having NCSs and could directly associate their condition to inflammatory state with markers like TNFR1 and hs-CRP. (Claassen *et al.*, 2014)

The clinical appearance of aSAH is highly altered by the patients' co-morbidity status. Severe congestive heart failure, COPD, pre-existing neurological disease and psychiatric conditions may complicate the initial assessment and definitely affect long-term outcome to the worse. It led to development of Charlson Co-morbidity Index (CCI) and its application on aSAH patients. (Boogaarts *et al.*, 2014) If this index had been available

at the time of study design, it would have been easier to adjust the outcome for co-morbidity.

Finally two facts need to be mentioned, which might have confounded some aspects of the results. A difference might have emerged between patients presenting directly at the University Hospital compared to those, being referred from other health facilities with the ambulance services. In the latter case we were compelled to seek secondary information on patients' initial status by consulting local hospital journals and ambulance records. These patients came frequently sedated, sometimes anaesthetised and in respirator as recommended by inter-hospital transport protocol based on current knowledge (Uren *et al.*, 2009). Neurological investigations were seriously influenced, often impeded on these patients.

Technical circumstances compelled us to exclude 12 patients, 3 of whom were foreign citizens, presumably not available for outcome investigations and in 9 cases, informed consents could not be obtained or were withdrawn. These cases might have affected outcome, as the main reason for failure to obtain consent was that these patients were severely affected by their illness and could not sign it themselves and relatives could not be traced. It might have been however compensated by those few, who withdrew their consent because they

were simply not interested in further blood samples, neurological investigations or/and an impending LP in an adjoining study.

III. Methodological considerations

When we designed these studies no data existed on many of the neuromarkers' kinetic properties. Some of them are fast markers (FABP, TNFR1, DDMR) increasing within hours, some even within minutes, others take days to rise (CRP). Even circadian rhythm is described in some of the markers (FABP) (Pelsers *et al.*, 1999). The admission sampling, which could fall on day0, day1 or day2 could be taken at any hour of the day, the following samples were taken meticulously the same hour every day. This fact could indicate a wider technical dispersion of the data on the initial days.

As in all patient related clinical studies, we also have data samples missing. In our case, the blood-sample collection's adherence to study protocol was around 87%, depending on the patient selection for the study, meaning that 13% of the blood values never reached the study register (not taken, sample foul, laboratory error). This led to the exclusion of 13 patients from Paper I due to difficulty in calculating the area under curve (AUC) in serial data. After publication, we have solved this problem, by using weighted (geometric) mean values for the time -series.

After recalculating the figures for the whole sample, it led to the same conclusion, only changing the results by the second decimal.

These studies were not aimed to parallel treatment strategies (surgical clipping vs. endovascular coiling) and these data were presented of pure descriptive reasons only, so it can be viewed in relation to other studies in the literature. However, out-of-study comparison showed similar results as published in much larger multicentre investigations, *e.g.* the ISAT or BRAT study. (Molyneux *et al.*, 2002; Spetzler *et al.*, 2015) For the sake of interest, data taken from Paper II show 28% and 30% dead or dependency, 46% and 41% detectable neurological deficit and 28% and 33% ADL assistance necessity for operated and coiled patients respectively at one-year follow up. None of the figures showed statistically secured difference, only a slight tendency to more focal deficit after surgery and slightly more assistance requirement after coiling. One could argue that the more severe the SAH (volume effect, intra-parenchymal component, herniation risk), the larger is the possibility for neurosurgical intervention (clipping), thus it would skew the outcome. However, this point is usually counteracted by the fact that the most elderly and patients with most severe co-morbidity tended to be treated endovascularly (coiling).

Before performing patient selection for study resulting in Paper I (CRP), we had some methodological discussions how we should relate to indwelling ventricular catheter or parenchymal pressure transducer insertion as a confounding factor in CRP elevation. We came to the conclusion that we confirm the fact that catheter-insertion procedures initiate an inflammatory reaction (Alhadad *et al.*, 2007; Almagor *et al.*, 2003) but not in the magnitude as in major operations inclusive craniotomy for cortical tumour operations (Mirzayan *et al.*, 2007). Although there is no literature support, it is plausible, that skull-base surgery as clipping an aneurysm results in an even larger inflammatory process than craniotomy for cortical tumours, thereby constituting an even more important confounder for CRP elevation. This fact is the most important criticism for previous CRP studies in SAH patients. Ventricular catheter or parenchymal pressure transducer were placed in patients, either in need of invasive intracranial pressure monitoring, CSF drainage, or both because of a SAH complication (hydrocephalus, DCI, CI). We decided that exclusion of patients with indwelling ventricular or parenchymal catheter (42% of study I. population) would result in a skewed inclusion of aSAH patients (only the very best of patients and the moribund ones) and that the results could no longer be applied for this patient group.

In Paper III, performing qPCR (quantitative real-time polymerase chain reaction) assay for rs1333040 SNP, the test failed and due to probe C_8766795_10 failure it could not be repeated. This SNP which is part of the cyclin-dependent kinase inhibitor 2 B-antisense RNA1 (CDKN2B-AS1) a non-coding RNA sequence, better known as ANRIL (antisense non-coding RNA in the INK4 locus), which has a function of silencing other genes in the proximity. (Popov and Gil, 2010). It is rather unfortunate that this particular assay failed as it was associated with atherosclerosis, cardiovascular diseases, diabetes type 2, and most of all intracranial aneurysms. (Abrantes *et al.*, 2015; Alg *et al.*, 2013).

Our follow-up, as I described in the introduction, is our pride, but one may argue that even longer monitoring would have been desirable (Molyneux *et al.*, 2015). Wilson and co-workers showed, however, that in the time-course of recovery, following poor-grade aneurysm in 88 patients, the largest improvement occurred between hospital discharge and 6 month (23.5%, which proved to be significant). Further recovery measured by modified Rankin scale, was 18% between 6 months and 1 year and further 19% between 1 year and 3 years. They did not differ significantly between each-other (Wilson *et al.*, 2013). Nylén pointed out the risks associated with extended follow-ups,

including increased number of drop-outs, changed social and financial status and other confounding circumstances which would cloud the disease-dependent outcome (Nylen, 2007). On the other hand, one cannot deny the fact, that the 37% progress in neurological recovery between 6 month and 3 years in the Wilson study, is a more substantial improvement than that of from discharge to 6 month (and statistically significant, if one after-calculates the figures). This is one of the reasons why very long-time (10-15 years) follow-up is under way in our aSAH patient -group. Another reason is to confirm a recent finding from Lindgren et al. that aSAH patients, even long time after their ictus, still die of cerebrovascular reasons, unlike the general population, where cardiovascular explanations are the most frequent causes of death (Lindgren *et al.*, 2015).

IV. Classification considerations

The diagnostic and severity scales detailed in the introduction (RLS85, H&H, GCS, WFNS, Fisher scale) are still valid today, although many of them are modified and numerous new ones enrich the collection. I give a brief summary of the proposed changes and the potentially new scales to use. The reason for this passionate interest in modifying the old scales or inventing new

ones lies in the fact that nearly all of them perform poorly in outcome predictions. There is an inherent conflict between designing and using a diagnostic scale. The easier, more user-friendly a scale is (less categories) the less mathematical chance it has for good outcome prediction. Conversely, the more detailed, more points / categories it has, the less popular it becomes but has the potential (at least mathematically) for better prediction. This ambiguity may contribute to the fact that new prediction models appear frequently in the literature, although none of them transferred to clinical practice (Jaja *et al.*, 2013).

A. Diagnostic scales

One of the modifications to the WFNS (m-WFNS) scale was suggested by Sano *et al.* by moving GCS 14 to WFNS II and GCS 13 to WFNS III, regardless of focal deficit. It eliminates the assessment on double axis, which makes the scale easier to use (Sano *et al.*, 2015).

SAH score from Naval and co-workers proposed a summary point system from GCS (1-4 points), age (1- 4 points), medical comorbidities (1- 3 points), which resulted in 9 categories and giving, according to the authors, superior prediction to WFNS (AUC=0.821 vs. 0.771). (Naval *et al.*, 2014).

MGH-score was developed in the Massachusetts General Hospital and was

based on a five-criterion system, awarding 0 or 1 point. The criteria were age (<50, >50 years), H&H (no coma I-III, coma IV-V), Fisher scale (1-2, 3-4), aneurysm size (<10, >10 mm), giant posterior circulation lesion (no, yes). Scores are from 0-5 (Ogilvy and Carter, 1998).

In his modified MGH-score (m-MGH), Lagares changed the level of consciousness to dichotomised WFNS scale (I, II-III, IV-V), otherwise similar to above and claimed to have better performance (Lagares *et al.*, 2005).

GCS grading system (GCS-GS) is compressed version of the GCS system, there GCS points is put into a 5-scale system. (15, 14-12, 11-9, 8-6, 5-3). It performed reportedly as well as WFNS and GCS.

NIS-SSS, Nationwide Inpatient Sample (American ICD-9 based administrative database) SAH Severity Score is developed for database demographic studies to control for SAH severity. Patient selection with the diagnosis codes for coma, stupor, hydrocephalus, paresis/plegia, aphasia and cranial-nerve defect as well as treatment codes for ventriculostomy, CSF shunt and mechanical ventilation were collected and univariate logistic regression coefficient calculated. NIS-SSS was derived from a weighted sum of these coefficients. It was validated in more than 100,000 patients and then performance tested. It performed better

than H&H for outcome prediction (Washington *et al.*, 2014).

The FOUR score, full outline of unresponsiveness score was developed after realising the shortcomings of the GCS system on brainstem reflexes. It gives 0-4 points in four functions; eye movement, motor response, brainstem reflexes (pupil, cornea, cough), and respiration. According to records, it performs similar to GCS (Chen *et al.*, 2013).

The HAIR score is a prediction model comprising of 4 attributes of SAH. It gives different points in different severity on a non-linear scale. H&H (I-III→0, IV→1, V→4), Age (<60→0, 60-80→1, >80→2), Intra-ventricular haemorrhage (IVH) (no→0, yes→1) and Re-bleeding (no→0, yes→1). Scores 0 –7 predict in-hospital mortality between 0.9 – 83% evenly distributed (Lee *et al.*, 2014).

Takagi *et al.* had an entirely mathematical approach of the allocation of grading based on the GCS. He was using combinatorial methods to divide the 15 points into 5 categories. The integrated differences led to the Grading Scale based on GCS (GSbGCS): I –15, II –14-11, III –10-8, IV –7-4, V –3. They claimed more symmetrical patient - distribution and better prediction performance than WFNS and they promised an easy adjustment if therapy results improved (Takagi *et al.*, 1999).

The radiological assessment scales have evolved as well over the years. Claassen suggested a modification of the Fisher scale (mod Fisher scale) after showing the importance of bilateral intra-ventricular hematoma in the risk of DCI development. His suggestion was: 0 – no SAH, 1 – thin SAH, no IVH in both ventr., 2 – thin SAH, IVH in both ventr., 3 – thick SAH(>1mm), no IVH in both ventr., 4 – thick SAH, IVH in both ventr (Claassen *et al.*, 2001).

A new scale of radiological classification was proposed by Wilson, the Barrow Neurological Institute SAH scale (BNI-SAH Scale). It abandoned the importance of intra-ventricular hematoma as a prognostic factor and concentrated entirely on the thickness of the SAH, measured perpendicular to the direction of cistern or fissure. The scale classified the SAH in 5 categories: 1 – No SAH, 2 – ≤ 5.0 mm, 3 – $>5-10$ mm, 4 – $> 10-15$ mm, 5 – >15 mm. The simplicity and Gauss distribution of patients in the scale are a huge advantage, but the scale needs validation in prospective studies (Wilson *et al.*, 2012).

It was finally relieving to read that the Charlson Comorbidity Index, where they allocate points by different coexisting diseases added no extra information in a SAH prognostic model (Boogaarts *et al.*, 2014).

B. Outcome scales

GOS, GOSE, NIHSS (although not strictly an outcome scale) and the Barthel Index, detailed in the Introduction, continue to serve as useful tools in outcome assessment. These scales can, and were in our research group (Nylen *et al.*, 2007), supplemented by the Mini Mental State Examination (MMSE), which can subdivide the patients with favourable outcome by testing their higher cognitive functions. It requires speech or communication and is designed to test orientation, memory and attention (Folstein *et al.*, 1975).

NIS-SOM, as in Nationwide Inpatient Sample SAH Outcome Measure, as its sister NIS-SSS mentioned above is an administrative tool to control demographic and economic studies' outcome and disease severity. It is derived from ICD-9 diagnose- and therapy codes and calculates regression coefficient-based measures of outcome. It is not for clinical use, but can be applied for hundreds of thousands of patients in databases (Washington *et al.*, 2014).

Finally, there is an outcome evaluation tool, which has gained wide acceptance in literature, and it might be the preferred assessment measure in the future; the modified Rankin scale (mRS). It reflects general disability in the sense of handicap rather than impairment. It is a six graded scale: 0 – no symptoms, 1 – no disability

some symptoms, 2 – slight disability (able not all, but the majority of activities), 3 – moderate disability (some help, able to walk), 4 – moderately severe disability (unable to walk or take care of body needs), 5 – severe disability (bedridden, incontinent, constant nursing). The scale is often dichotomised to favourable outcome (mRS 0-2) and dependent (mRS 3-5) (van Swieten *et al.*, 1988).

V. Remarks on genetic markers

A. ApoE

Apolipoprotein E is a major cholesterol transporter and an active regulator of lipoprotein metabolism in the body (Siest *et al.*, 1995), and it is understandable that genetical variants coding different protein alternates have different effectivity in these mechanisms. Apart from role as a transporter in the periphery, it also assists neural transmission (Mauch *et al.*, 2001) and in pathological conditions ApoE has been suggested to promote aggregation of amyloid β ($A\beta$) into plaques, which are characteristic of Alzheimer's disease.

The findings in our study in Paper III are fairly clear-cut. In our clinical material, we found no association of the variant allele *APOE ϵ 4*, either with the incidence of aSAH compared in a case control study or in an

aSAH patient cohort study on CVS and long term-outcome. The results were so unambiguous, that if we 10-doubled the patient number in our material with the same dispersion of data, it would still lead to the same conclusion. As this question has been extensively researched even at this diagnostic entity, why is it still interesting?

The answer lies on at least two levels. Firstly, the published studies demonstrate a surprisingly unequivocal conclusion, with approximately the same number presenting data for a negative effect of the altered allele on the outcome (Niskakangas *et al.*, 2001; Tang *et al.*, 2003; Leung *et al.*, 2002) as demonstrating non-significant effect or no influence at all (Morris *et al.*, 2004; Fontanella *et al.*, 2007). One would think that meta-analysis could solve this discrepancy by collecting all data and calculating summarised-odds for this allele's effect on several hundreds or thousands of patients. Unfortunately it is not always true, as those two recently published meta-analyses on this subject demonstrate (Lanternia *et al.*, 2007; McColgan *et al.*, 2010). They present equally conflicting conclusions, on the basis of, and it is similarly intriguing, only partially overlapping study-material. My interpretation of the current scientific opinion is that it is rather unclear on this matter, so I hope that I pushed the debate a little further.

Secondly, the frequency of genetic variation on *APOE* allele demonstrate a wide geographical dispersion on the whole planet (Gerdes, 2003) in Europe (Fontanella *et al.*, 2007) and especially in Northern-Europe (Lindekleiv *et al.*, 2010; Juvela *et al.*, 2009; Kern *et al.*, 2015). The allele frequencies vary from the highest, 41% among pygmies in Africa to the lowest 5% in Sardinians. In Europe, there is clear South-North distribution from 5-6% in Sardinians and Greeks to 31% in the Lapps in the Northern regions of Scandinavia (Corbo and Scacchi, 1999). This fact may influence how we interpret the results, found in another part of the world or even a neighbouring country and how we can apply this knowledge on our patients.

One new aspect has emerged recently in the literature. Apart from the sheer existence of an altered allele there might be other factors influencing its expression, transcription and action. Two Chinese studies looked at the polymorphism of promoter gene to *APOE*, as a probable factor influencing apoE protein expression and found that -219T allele, compared to -219G was negatively associated with both CVS and re-bleeding after aSAH (Wu *et al.*, 2010; Yin *et al.*, 2015).

B. 9p21

The approximately 400 kbp large nucleotide sequence found on the short arm of the 9th chromosome in the human genome has been in the focus of genetic studies for three and a half decade and particularly genetic association studies, investigating vascular anomalies for the past 10 years. The intensity of research can be described with the hit result of 1831 articles published with the single search word: 9p21.

In the investigated region we could identify six single nucleotide polymorphisms, SNPs, which had the potential to correlate to aneurysm rupture between an aSAH patient cohort and controls. We added an extra SNP, (rs1537378) which had been associated previously with ischemic stroke variant; large vessel disease (Gschwendtner *et al.*, 2009). Unfortunately one SNP's (rs133340) assay failed, as discussed above. One SNPs (rs10757278) distribution showed significant correlation between samples and controls. The univariable regression analysis showed significant ORs with two SNPs, but only the above mentioned SNP stayed significant, when we controlled the model for hypertension and smoking as further risk factors for aSAH.

The identified SNP is included in a DNA code sequence, determining non-coding RNA structure in proximity of cyclin-dependent kinas inhibitor 2A and B (CDKN2A & B)

coding allele. It has been associated with most of all myocardial infarction (Fan *et al.*, 2013), but as the main function of this RNA sequence is to suppress other gene-transcriptions, its attenuated function favours different tumour growth (Holdt *et al.*, 2013). Subsequently many tumour types (melanoma, lymphatic leukaemia, oesophagus carcinoma, lung cancer, bladder cancer) have been associated with this anomaly (Lee *et al.*, 2015).

What is more interesting in an intracranial vessel-rupture point of view is that it is strongly associated with arterial stiffness (Phababpha *et al.*, 2013), carotid artery stenosis (Bayoglu *et al.*, 2014), intracranial arteriovenous vessel-malformation (Bendjilali *et al.*, 2014) and through CARD8 expression ANRIL increases stroke risk by promoting atherosclerosis (Bai *et al.*, 2014; Kojima *et al.*, 2014).

The risk allele in the SNP is the G variant, the homozygote wild type, AA reduces risk to MI and intracranial aneurysm formation to OR: 0.77, heterozygote altered gene, AG increases risk to OR: 1.3 and homozygote, GG to OR: 1.6 (Helgadottir *et al.*, 2008). A recent study from Japan explored the predisposing genes to aneurysm rupture (Nakaoka *et al.*, 2014). They compared the gene expression profiles from 8 ruptured, 5 unruptured IAs and 10 normal intracranial vessel-walls. They found 2 distinctive

clusters in the ruptured IA group (early-late). In comparison with the normal cluster, they described 430 genes which were upregulated, mostly inflammatory, immuno-response and phagocytosis regulating genes (S100, calgranulin) and 617 genes which were down-regulated. Among these, they described ANRIL and Kruppel-like family of transcription factors which were interpreted as mechanical weakness in the vessel wall, as these genes had anti-inflammatory properties.

If one thinks that our life is destined and our risk profile to diseases is genetically predetermined, one can find some consolation in Hindy's study. He investigated if an SNP, known to have close association to cardio-vascular diseases (CVD) in the 9p21 region, can be affected by environmental factors. He tested in a large cohort, whether wine and vegetable diet can influence the expression of rs4977574 allele and thereby alter disease incidence. He did find a reduction of ORs to CVD and an interaction of the SNP's expression, modifying HDL cholesterol levels.

VI. Remarks on biochemical neuromarkers

Biomarkers are qualitative and quantitative biological substances or characteristics, which define pathological conditions and may give indication on disease severity and type of therapy (Mouhieddine *et al.*, 2015). I

endeavour to summarise below, if and how the substances we analysed fulfil the above criteria.

A. BDNF, NSE, GFAP

Neuromarkers above, closely described in the Introduction, have surprisingly not shown association with either CVS or any of the outcome parameters. There could be several reasons for the negative results, as all three markers have been described to increase in stroke (Siironen *et al.*, 2007; Anand and Stead, 2005; Nylen *et al.*, 2007) and after traumatic brain injury (Failla *et al.*, 2015; Cheng *et al.*, 2014; Nylen *et al.*, 2006).

We could see a negative association (although not significant) between BDNF and initial neurology, as is conceivable with a neurotrophic protein. It could be explained by the fact, that milder injury initiates neuronal reparation and apoptotic mechanisms, while a large haemorrhage instigates necrotic processes. BDNF is described to bind to specific Trk β membrane receptors and the non-specific soluble p75 receptors. Via both mechanisms it can activate NF κ β enzyme-system which leads to apoptosis.

NSE is a promising biomarker, as it not only can reflect the extent of neuronal injury, but also persevere on a higher level mirroring a persisting secondary ischemia. (Cheng *et al.*, 2014) However there are several limitations

in its use. NSE is affected profoundly by haemolysis, as erythrocytes contain a large amount of it. Furthermore, trauma patients, without head injury exhibited NSE increase not leading to neurological symptoms. Ischemia in abdominal organs in rodents has been documented to increase NSE (Pelinka *et al.*, 2005). These facts have to be taken into consideration when interpreting NSE results, especially from TBI.

It was rather dissatisfying to detect the lack of association to outcome in GFAP samples, as GFAP is one of the most encouraging neuro-injury markers with astrocyte/glia specificity. We encountered generally low levels in the whole sample, not unknown in literature (Mayer *et al.*, 2013), nevertheless after scrutinising the data we found that the initial levels of GFAP showed a sharp rise, which normalised until day 3. The worse the patient's initial neurology was, the higher the GFAP mean values reached underlining the fast-reacting character of this neuromarker. It even showed a secondary rise in moribund patients, corresponding to earlier findings in our group (Nylen *et al.*, 2007).

The reason we could not find an association with these markers may include the fact, that the disruption of the blood-brain barrier may have been different even within groups or that the markers leaked out to serum in an inconsistent pattern and/or inadequate concentration (Olivecrona and Koskinen,

2012; Jung *et al.*, 2013). Furthermore, the study of Paper IV was not designed or powered to investigate any particular marker. It was rather a feasibility study to test a new array technique in a new patient population.

B. CRP

We have proved in Paper I that CRP increases after subarachnoid haemorrhage in endovascularly treated patients with a continuously rising pattern, independently from confounding factors. The course of CRP showed stronger correlation to outcome parameters than initial neurology or radiological evaluation. CRP's reaction on SAH is not novel as it has been demonstrated since 2003 (Di Napoli and Papa, 2003) and repeated in different patient populations ever since (Feigin and Findlay, 2006; Rothoerl *et al.*, 2006; Fountas *et al.*, 2009; Jeon *et al.*, 2012; Juvela *et al.*, 2012; Romero *et al.*, 2012; Hwang *et al.*, 2013; Romero *et al.*, 2014; Badjatia *et al.*, 2015). The unique aspect in our study is that we could eliminate one major confounding factor (craniotomy) and could ascertain a strong enough association between an early reaction of the marker and outcome to provide a prognostic curve. There are thousands of articles to prove that inflammatory processes are initiated immediately after the bleeding and most likely these reactions are more important to follow than neural damage mechanisms, as the inflammation always

precedes neural injury and could potentially be reversed (Chalouhi *et al.*, 2012).

The initial theory was to correlate the course of CRP during the first 8 days (weighted mean) to outcome and it was a surprise, that not only the maximum value, but as early as on day 2, CRP could be used as independent predictor, with nearly the strength of the entire series. This finding could be clinically applicable, especially in unconscious, sedated patients who are not available for neurological examination.

In Paper IV, we tested CRP again in a selection of patients as part of a validating process for a novel multi-array system. As the array was primarily designed for ischemic stroke detection, they employed high-sensitivity CRP, which proved to be too sensitive for the intense inflammatory reaction after SAH. As we described in Paper I, CRP increased to a median level of 53 mg/ml, which exceeded the upper level of the hsCRP test in many samples. Despite this fact, it showed good prediction values in accordance with the results of Paper I. (Some samples however, may have appeared in both studies.)

C. IL-6, TNFR1, NGAL

All the inflammation markers on this neuropanel showed significant correlation to outcome parameters, CVS development and except TNFR1 even to admission neurology

status. There is, however, a temporal shift in reaction among the markers and the association to different pathological events during the course of the disease may help to identify in what order in the inflammation cascade they are activated. In other words they reveal how fast the different markers react to stimuli.

While TNFR1 demonstrated a continuously rising pattern throughout the study period, doubling in concentration in the unfavourable patient-group, the other markers (IL-6, CRP, NGAL) showed a distinctive peak round day 3. CRP and IL-6 followed a one-peak pattern,

and persisted on a level many times higher on day 11-14 compared to normal (day 0). NGAL showed however a double-peaked curve, especially in the unfavourable group, with a second top on day 8. It appears that this second increase coincides with the time-frame of CVS development. As it happens, CVS correlates very closely to poor outcome in this test-cohort.

In many aspects the brain's reaction to trauma, stroke or haemorrhage is similar. Either ischemia initiates injury (stroke,

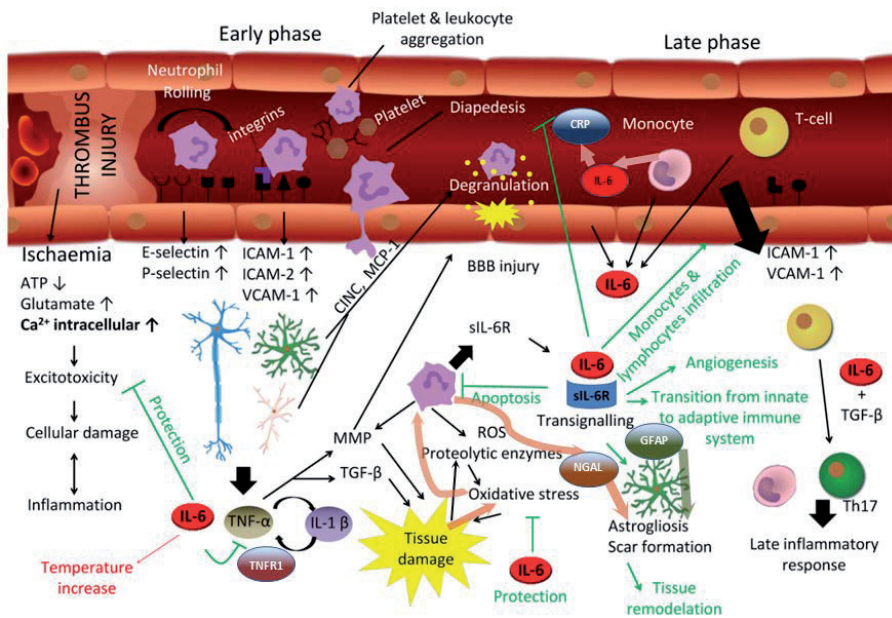


Figure 28 Part of the neuroinflammatory process, with special reference on the neuropanel's inflammatory biomarkers (CRP, IL-6, TNFR1, NGAL. Modified after Erta, 2012

although IL-6 returned to base-line, even in the unfavourable group by day 6. CRP, despite its peak on day 3, decreased slowly

hypoxia) or injury causes ischemia (haemorrhage, trauma) which again aggravates the injury (Erta *et al.*, 2012).

Hypoxia induces a biochemical cascade, involving excitotoxicity, oxidative stress, leading to apoptosis. (Fig. 28) Initially the neutrophil leucocytes extravasate after engaging in rolling, activation and transmigration. They release freely soluble IL-6 receptors (sIL-6R), which attract their ligands produced by damaged cells, together with TNF α and its soluble receptor TNFR1 and IL-1 β (Probert *et al.*, 2000). These cytokines attract leucocytes to the damaged area and facilitate their degranulation, contributing to cell necrosis. These relatively small inflammatory substances (20-30 kDa) can easily penetrate to the circulation, either through a disrupted BBB (sharp and pronounced rise) or through the glymphatic system (delayed and prolonged expression) and activate other immuno-inflammatory mechanism, like CRP excretion (Muroi *et al.*, 2011). IL-6, among others has a negative feed-back role in the necrotic and apoptotic processes by down-regulating TGF β , and metalloproteases, inhibiting neutrophil diapedesis and converting the innate immunological processes to adaptive, through T-cell recruitment (Erta *et al.*, 2012). It even promotes astrogliosis via NGAL, which contributes to microglia proliferation and transforming the astrocytes' GFAP structure towards tissue remodelling and endothelial cells to stimulate angiogenesis (Yang and Wang, 2015).

D. FABP, DDMR

Both FABP and DDMR had excellent correlation from the initial neurological condition through the CVS development and finally to all the outcome measurements. It indicates that both of them are part of highly sensitive and fast-reacting mechanisms and are interesting candidates for further neuromarker investigations. This rapid response quality is supported by previous findings (Fassbender *et al.*, 1997; Peltonen *et al.*, 1997).

Fatty-acid transporting protein (FABP) in our samples had high initial values, decreasing rapidly and reaching its nadir on day 2 post ictal. It is contradictory to findings of others, as it was described in 22 patients with stroke that values peak after 24-48 h (Zimmermann-Ivol *et al.*, 2004). It cannot be explained with a wash out effect during an operation, as only 7% of the poor-grade SAH patients were operated in this cohort. The rest of the patients were treated by coil-embolisation. Thereafter the FABP serum levels increased to a second peak on day 8, nonetheless merely in the unfavourable group, leaving the patients with favourable properties with normal values. As CVS correlates, as mentioned earlier closely to all the outcome measures, FABP separates the poor and favourable patient groups distinctively. This assigns bordering excellent prognostic properties both for DCI development and

outcome by all the scales (AUC_{ROC} 0.80 - 0.85). It is interesting to note the covariation of the nadir of this biomarker with that of DDMR, which I discuss below.

DDMR, a coagulation remnant is the other biomarker with excellent correlation to admission neurological status, demonstrating that the coagulo-fibrinolytic system is, actually the most rapid responsive mechanism in the body (Gruen *et al.*, 2012). It is particularly important as it was suggested that microthrombosis was responsible for DCI the most traitorous complication of aSAH (Vergouwen *et al.*, 2008). In a previous study, the elevated level of DDMR measured on the first day after aSAH, was found in good correlation to 6 month outcome (Peltonen *et al.*, 1997). In our study, the DDMR's initial values were four times the normal values, corresponding to the previously mentioned study. It fell rapidly to normal level until day 2, when it reached its nadir. Thereafter the level increased again to a plateau circa six times higher in the unfavourable group and three times above normal level in the favourable

group where it stayed during the rest of the observation period of 11-14 days. Because of the clear difference between the two groups, DDMR is proved capable of predicting outcome.

The concavity in the course of DDMR may be explained by the local routine use of iv. fibrinolytic agent, tranexamic acid, which is administered from the time of establishing a diagnosis until the aneurysm is secured (often within 24 h from admission) (Hillman *et al.*, 2002). After the drug effect diminishes the fibrinolytic activity returns. Unsurprisingly, the DDMR level reflects the amount of blood in the haemorrhage, thereby the assumed mass-effect, although as it is coagulation specific, it cannot differentiate between localisation and thence the neurological consequence. As there are both positive studies (Fujii *et al.*, 1997; Juvela and Siironen, 2006) and negative findings (Tseng *et al.*, 2007) in the literature, a recent review article considers the evidence for using DDMR as a biomarker after aSAH is weak and inconsistent (Boluijt *et al.*, 2015).

CONCLUSION

- We have established a pattern of CRP development during the first week after an aSAH, which was independent from the patients' infectious status.
- We could demonstrate a difference in this pattern between the patients with favourable and unfavourable disease development (complication, outcome).
- We could correlate this difference in CRP development to outcome measures and build a prognostic model to predict long-term outcome.
- In search for an even earlier predictor, we could identify CRP values already on day 2 with nearly the same predictive strength as the whole weeks CRP development and in parity with initial neurology and superior to initial radiological assessment in endovascularly treated patients.
- We demonstrated that Apolipoprotein E's genetical polymorphism had no influence either on the incidence of aSAH, the complication development or the outcome after the disease in West Sweden.
- We identified however, a single nucleotide polymorphism (SNP) on the 9th chromosome, which does seem to influence the incidence of intracranial aneurysm rupture in the West-Swedish population.
- Finally, we have tested a novel biochip array neuropanel in a selection of aSAH patients and found that six of the nine neuromarkers correlated to cerebral vasospasm and outcome. Four of them could be used for prediction purposes, thereby proving this methods usefulness in aSAH patient monitoring.

FUTURE PERSPECTIVES

These studies were aimed to increase our knowledge on some biochemical markers and genetical predispositions during the course of subarachnoid haemorrhage and test their usefulness in clinical settings.

We have demonstrated the applicability of inflammation markers in general and CRP in particular, not only as a marker of infection, but more as an assessment of disease severity. These markers, together with specialised tissue factors (here neuromarkers) may help guide clinicians in decision making on adjusting monitoring, choosing between therapy options or planning rehabilitation alternatives.

We have found a need for sequential sample-taking to follow the course of the disease or detect secondary events. It increases the credibility of the marker and reduces the possibility for confounding errors. With some markers, continuous or near-continuous sample-taking and measurements are available (*e.g.* microdialysis), with others it is on its way.

Multiple biochip-array technique is one of these techniques which can provide custom-made investigating tools to diagnose and to follow different kinds of pathological processes, including aSAH, using a drop of blood, liquor or any other body fluid.

Finally, we have added a piece to the mounting evidence demonstrating the connections between genetic inheritance and risk for aSAH. Mapping our genetical risk background is in the near future and it may affect our life-style, diet and prevention efforts to overcome our genetical fate and to modify this predisposition. It is already a reality with some oncological therapy decisions. The genetical predisposition may determine our preference in future therapy-choices in many more diseases.

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