Clincal Aspects of Biological Brain Damage Markers

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CLINICAL ASPECTS OF BIOCHEMICAL BRAIN DAMAGE MARKERS

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This thesis is dedicated to my family

The most beautiful thing we can experience is the mysterious.

It is the source of all art and science

Albert Einstein (1879 – 1955)

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Papers

This thesis is based upon the following scientific papers:

I **Unden J**, Christensson B, Bellner J, Alling C, Romner B *Serum S100B levels in patients with cerebral and extracerebral infectious disease.* Scand J Infect Dis. 2004;36(1):10-3.

II **Unden J**, Bellner J, Eneroth M, Alling C, Ingebrigtsen T, Romner B. *Raised serum S100B levels after acute bone fractures without cerebral injury.* J Trauma. 2005 Jan;58(1):59-61.

III **Unden J**, Bellner J, Reinstrup P, Romner B. *Serial S100B levels before, during and after cerebral herniation.* Br J Neurosurg. 2004 Jun;18(3):277-80.

IV **Unden J**, Bellner J, Astrand R, Romner B. *Serum S100B levels in patients with epidural haematomas.* Br J Neurosurg. 2005 Feb;19(1):43-5.

V **Unden J**, Astrand R, Waterloo K, Ingebrigtsen T, Bellner J, Reinstrup P, Andsberg G, Romner B. *Clinical significance of serum S100B levels in neurointensive care.* Submitted.

VI **Unden J**, Strandberg K, Malm J, Danielsson E, Rosengren L, Stenflo J, Norrving B, Romner B, Lindgren A, Andsberg G. *Biomarkers for coagulation system activation and brain damage in acute stroke differentiation.* Submitted.

Abbreviations

CK-MB	*Creatine Kinase MB*
CRP	*C - reactive protein*
BBB	*Blood Brain Barrier*
MRI	*Magnetic Resonance Imaging*
PCS	*Post-Concussion Syndrome*
MHI	*Minor Head Injury*
CT	*Computed Tomography*
GFAP	*Glial Fibrillary Acidic Protein*
NSE	*Neurone Specific Enolase*
MBP	*Myelin Basic Protein*
CK-BB	*Creatine Kinase BB*
APC-PCI	*Activated protein C-protein C inhibitor*
CNS	*Central Nervous System*
NF	*Neurofilament*
GOS	*Glasgow Outcome Scale*
GCS	*Glasgow Come Scale*
RLS	*Reaction Level Scale*
CVI	*Cerebrovascular Disease*
Spp	*Species*
Sp	*Specificity*
Se	*Sensitivity*
NPV	*Negative Predictive Value*
PPV	*Positive Predictive Value*
NICU	*Neuro-Intensvie Care Unit*
ICH	*Intracerebral Haemorrhage*
LACS	*Lacunar Syndromes*
TACS	*Total Anterior Circulation Syndromes*
PACS	*Partial Anterior Circulation Syndromes*
POCS	*Posterior Circulation Syndromes*
CSF	*Cerberospinal Fluid*
LDH	*Lactate Dehydrogenase*
GOT	*Glutamic Oxaloacetic Transaminase*
kDa	*Kilo-Dalton*
FABP	*Fatty Acid Binding Protein*

Background

Within almost all areas and specialities of clinical medicine exist diagnostic and/or prognostic blood tests, also known as biomarkers. Management and treatment of cardiac ischemia is largely governed by the results of one or more of the so-called heart specific biomarkers; Troponine T, Troponine I and CK-MB. Measuring blood levels of CRP and white blood cell count heavily influences diagnosis and management of infectious disease. Other blood tests such as creatinine, thyroid tests, hormone tests, liver enzymes, coagulation tests, neoplastic blood tests and blood gas analysis all play integral roles in current clinical practice. A treating physician would be lost without these tests due to the dependency that has been generated for these relatively simple and reliable clinical tools. In 1983, Bakay and Ward[6] summarised the optimal properties that an ideal brain biomarker should have. It should show high specificity for the brain, high sensitivity for brain injury, be released only after irreversible destruction of brain tissue, have a rapid appearance in serum, and be released in a time-locked sequence with the injury. The age- and sex-related variability should be low and reliable assays for immediate analysis should be available. Finally, and most importantly, a brain biomarker should show clinical significance.

The brain had eluded the technological development of biomarkers for some time. There is still no blood test available in clinical practice that reflects dysfunction or damage to cells of the nervous system. This is at first surprising, considering the caution and clinical respect that physicians show for brain disease. However, this same respect induces a higher standard for a brain biomarker, when compared to other organ systems. Also, the brain is physiologically very different to other organs in the body. The BBB is a selective barrier, hindering most molecules from passing over its tightly maintained integrity. Also, the brain is far more a qualitative organ than a quantitative one. Classic biomarkers used clinically today in other organ systems are quantitative in nature. This aspect confines primarily the prognostic ability of a theoretical quantitative brain biomarker.

Nether less, work started as early as the 1960´s[18] with studies presenting possible biomarkers for brain injury. These, however, failed to show any clinical utility. During

the following 25 years, more encouraging reports on serum and CSF levels of other biomarkers such as CK-BB[141], LDH[6,47,74] and GOT[80] were published. These markers also failed to show enough clinical relevance and were therefore gradually discarded as potential biomarkers for the CNS.

Potential brain biomarkers

During the last 10-15 years, brain damage biomarker research has accelerated. Most of the work has implicated a protein known as S-100 as a promising surrogate marker for the brain. S-100 was first described by Moore in 1965, achieving the name S-100 for its solubility in 100% saturated ammonium sulphate at neutral pH[91]. S-100 is an astroglial protein of about 21 kDa. Although the specific function of the protein has not been established, it seems to have both intracellular and extracellular effects[2,24], primarily through it's calcium binding abilities. It exists in vivo as a dimer combination of two subunits, consisting of α and/or β chains. Many variations of these subunits have today been identified but it seems that the ß-subunit is most specific for brain tissue. Due to the importance of the ß-subunit when referring to brain tissue, S-100 is often referred to as S100B. The biomedical industry soon developed reliable assays for detection and quantitative measurement of S100B.

S100B is eliminated from the body by renal metabolism and urinary excretion[168]. The half-life is short; recent data suggests a half-life of less than 30 minutes[73]. The age- and sex-related variability has been shown to be low[159]. S100B can be measured in serum, CSF, urine[40], amniotic fluid[39] and saliva and has been shown to be very stable in a variety of conditions[116]. Our group has recently established successful measurement of S100B in capillary blood (unpublished work, *Astrand R et al* 2006); a relevant aspect when considering paediatric usages of biomarkers. Basic research efforts enabled further work concentrating on clinically relevant situations and S100B as a brain damage marker.

Other markers have not been studied as extensively as S100B although several have shown interesting preliminary results. GFAP and NSE are such examples which have been reported in the literature.

GFAP is also an astroglial protein, representing the major part of the cytoskeleton of astrocytes[28]. It has a molecular mass of between 40 and 53 kDa and was first isolated in 1971 by Eng et al[27]. The major problem concerning this marker has been the lack of a reliable commercially available assay.

NSE was described by Moore in 1965[91], has a molecular mass of 78 kDa and a biological half-life of over 20 hours[65]. NSE is used clinically as a tumour marker (such as small-cell lung cancer, neuroblastoma and melanoma). The protein is, however, also released by haemolysis which may be a clinical source of error[65,102].

The remaining markers that have been studied, such as MBP[146,153,167], tau protein[16,19,37,146,170], FABP[105,106,166,171] and spectrin breakdown products[78,109,110,123] show some encouraging preliminary results although more work is necessary in order to draw more concrete conclusions.

The focus of this thesis will therefore be concentrated on S100B, GFAP and NSE; the potential biomarkers with the most promising results.

Minor head injury

This trauma mechanism presents several clinical difficulties. Management often involves neuroimaging (particularly CT) and/or in-hospital observation. Guidelines are today largely based upon patient history, specifically occurrence and duration of unconsciousness and/or amnesia, as well as clinical examination. All these points can prove logistically difficult. The head trauma alone is often associated with a degree of acute cognitive dysfunction. Children, elderly and intoxicated patients are common in this trauma category; all of which are difficult to examine and interview with clinical precision. Concerning management, CT procedures involve practical difficulties and observation is costly[59]. Recently, work has been presented implicating electromagnetic radiation from repeated CT procedures as potentially harmful[45]. Despite normal CT scans, many patients experience long-term neuropsychological symptoms (PCS) that cannot be predicted with today's clinical routines[14].

Therefore, a reliable brain damage biomarker in this setting is welcomed and, considering the logistical aspects of a potential biomarker in this setting, a serum marker is warranted.

Early results from Ingebrigtsen *et al* showed a very high negative predictive value for intracerebral lesions, using MRI as golden standard, and presented promising results referring to PCS occurrence prediction[60-63,125,158]. Since these studies, other groups have confirmed the findings and advanced further in the field[9,10,50,95,133,135,142,143,154,155]. It has been suggested that S100B might in fact be superior to existing gold standard techniques, such as CT and MRI[8]. One group has, however, not seen these promising results[21]. A proposed weakness concerning S100B has been a lack of brain specificity[4] although the biomarker does seem to have very high brain sensitivity. Two large studies that were recently completed illustrate the potential ability of S100B to effect management of minor head injury[12] (and *Muller K et al*, submitted 2006).

The epidural haematoma is the primary reason for management routines, such as CT and/or hospital admission, after acute head injury. This clinical phenomenon has some theoretical issues, when considering detection of the condition by biomarkers. The epidural haematoma is essentially a head injury and not a brain injury, at least not in the early temporal phases of the disease. The question was whether or not a brain biomarker could detect this feared condition after head injury. This aspect has not been examined.

Concerning other potential biomarkers, only a few studies exist and these concern NSE[7,9,10,29,92,139,142,143]. The results are not as promising as for S100B[128,167].

Severe head injury

Severe head injury and neurointensive care pose certain clinical problems. Outcome prediction can be difficult to predict with existing parameters; an aspect where biomarkers of brain injury may be useful. The prediction of secondary complications in a neurointensive care patient is also an interesting application of a

potential brain biomarker. These patients are often unconscious and/or sedated, with external mechanical ventilation, which makes traditional clinical evaluation difficult.

Research concerning biomarkers in severe head injury has again focused on the S100B protein and primarily been concentrated on outcome prediction, showing favourable results[23,54,92,111,113-115,117,118,120,122,132,133,156,161,162,164]. Results have also shown correlation to presence and size of cerebral contusions[54,125]. S100B had also been shown to be correlated with secondary neurological complications in critically ill neurointensive care patients[117].

Data involving serum GFAP levels has also been presented; also showing good correlations to outcome scores[89,99,164]. NSE has also been investigated[50,87,111,113,114,128,161,164] although the findings were consistently not as convincing as with GFAP and, primarily, S100B.

Problems with the biomarker approach for these patients include, as with minor head injury, specificity issues[4]. The study by *Anderson et al* was however criticised[48,112,124,131]; mainly for the methodological exclusion of possible head injury in the patient material. With respect to outcome, a worry has been the ability of an essentially quantitative biomarker to predict outcome in a very qualitative organ such as the brain. Prediction of secondary neurological complications has been favourably reported in one study[117], but more work needs to be produced in order to fully understand the dynamics of biomarkers, such as S100B, in this setting.

Ischemic stroke

Advancements in treatment possibilities of both haemorrhagic and ischemic stroke have put pressure on swift and accurate diagnostic capabilities. Thrombolysis is today the treatment of choice for embolic ischemic stroke, although novel neuroprotective therapies have been suggested[26]. Clinicians must therefore quickly, with very high reliability, rule out haemorrhagic stroke before initialising treatment. This is today done with neuroimaging, primarily CT, which is time-consuming, can be logistically difficult to perform and does not allow for pre-hospital diagnostics. Outcome prediction of stroke is also a clinical challenge, for instance when deciding

on the level of post-stroke care necessity. In critically ill stroke patients, neurointensive monitoring is important but difficult with today's technology.

Many studies have examined biomarker levels, predominately S100B, GFAP and NSE, in stroke. These studies show encouraging results with biomarker level relationships to stroke size and outcome[1,5,17,20,34,35,55,86,89,165]. One recent study showed S100B to be a strong diagnostic indicator of stroke in a study considering 26 different potential biomarkers[83]. However, little research has explored the ability of potential biomarkers to differentiate stroke subtypes. This is today the most important diagnostic application of a brain biomarker referring to this clinical condition.

Infectious diseases of the CNS

Infectious diseases in the brain, such as viral encephalitis and bacterial meningitis, are feared diagnoses. Rapid treatment is often essential in order to eliminate the infectious agent and ensure good outcome. Diagnosis is sometimes difficult, as many CNS infections have unspecific symptoms, especially in the early phases of the disease. Management often involves treating patients with antiviral and/or antibacterial agents before a diagnosis can be made.

A few studies have implicated the possibility of a brain damage biomarker in this setting, as a surrogate marker for diagnosis and prognosis of brain infections[11,25,30,42,57,58,79,138,151]. However, these studies included few patients, utilised biomarkers now known to be insufficient, used older analysis kits or sampled CSF (which is generally impractical).

Other clinical areas

Biomarkers of brain damage have also been studied in subarachnoid haemorrhage[46,100,107,152,160] and global anoxia after cardiac arrest, showing interesting results referring to outcome prediction[15,36,85,86,88,93,108,126]. Biomarkers have also been analysed peroperatively in different surgery types, such as coronary bypass surgery[3,13,31,52,65-73,157], carotid surgery[22,38,97,121] and general surgery[56,64,81,82], as a

measure of possible per-operative brain damage. These results have been mixed, primarily due to contamination and specificity issues. Finally, there exist studies within other areas of medicine, including electroconvulsive therapy[106,169], schizophrenia and depression disorders[129,130], sporting events[96,101,143-145], brain tumours[75,163] and hypoxic neonatal encephalopathy [41].

Summarising, many studies have looked at different neurological diseases in search of a reliable and robust brain damage biomarker. The areas of traumatic brain injury, foremost MHI, as well as stroke and brain monitoring seem to be in most desperate need of a novel brain damage marker. However, more scientific material is necessary in order to fully validate these biomarkers if clinical practice is to become a reality.

CSF sampling is generally impractical and the aim of a biomarker for brain damage is to facilitate clinical practice, not complicate it. The primary medium to be considered must therefore be blood (or serum).

S100B was by far the most interesting of the potential biomarkers, considering the published material. The most pressing problem was the issue of poor brain specificity of S100B. It is known that this protein is found in other organs than the brain[44,65,76,77], but the actual clinical significance of this was assumed to be negligible.

GFAP may also be of interest, although the information concerning this marker is limited, mainly due to the lack of reliable commercial assays. Other markers have either failed to show promising results, such as NSE and CK-BB, or are too premature to draw solid conclusions about, such as NF and spectrin breakdown products.

Aim of the thesis

Although many studies seem to show that biomarkers, such as S100B, are promising measures of brain damage, there exist several problems which must be solved before clinical practice can be considered.

Most importantly, the clinical specificity issue of S100B requires special investigative attention. Although the sensitivity has seldom been criticised in the literature and assumed to be very high for S100B, this also craves further analysis. Furthermore, other clinical applications of biomarkers such as S100B need to be examined.

The primary aims of this thesis are therefore:

- To examine the specificity of serum S100B in clinically relevant situations.
- To examine the sensitivity of serum S100B in head injury.
- To investigate serum S100B levels in infectious disease.
- To investigate the source of serum S100B in patients with multitrauma and brain injuries.
- To examine the clinical utility potential for serum S100B in neurointensive care.
- To investigate the ability of a biomarker panel to differentiate ischemic from haemorrhagic acute stroke.

Study design and methods

In order to satisfy the aims of the thesis several projects were designed. These were all clinical in nature and all examined serum levels of biomarkers. A total of 294 patients form the clinical material for this thesis. None of the patients are included in more than one study.

Biomarker analysis

S100B analysis used the same system for all the studies included in this thesis. S100B was analysed using a fully automated LIAISON® system (AB DiaSorin, Bromma, Sweden.). This system detects the β subunit of the S100 protein, with the analytical sensitivity of 0.013 µg/l. The typical intra-assay and inter-assay precision is below 5% and 10%, respectively. The cut-off value has been found to be 0.15 µg/L for the 95th percentile referring to healthy blood donors, according to the manufacturer.

For NSE detection, the fully automated LIAISON® system (AB DiaSorin, Bromma, Sweden.) was also used. An immunoluminometric assay is used measuring levels between 0.04 – 200 µg/L with an intra-assay and inter-assay precision below 2% and 6%, respectively. Serum levels in excess of 10 µg/L are considered to be pathological[98].

GFAP was analysed using a modified sandwich ELISA that has previously been described[127]. The upper reference level has been reported to be 33 ng/L representing the 97.5th percentile of healthy subjects[90]. Values below the measurable cut-off of 30 ng/L were given the value 29 ng/L for logistical ease (see Paper VI).

APC-PCI complex concentration was measured using the previously described DELFIA assay, which has a functional sensitivity in Stabilyte-plasma of 0.032 µg/L. Using Stabilyte tubes, the concentration in healthy individuals was 0.07 to 0.26 µg/L with a mean and median of 0.13 µg/L[147-150]. The within-run coefficient of variation was 4.8% at 0.15 µg/L and 3.2 % at 0.40 µg/L, while the between-run coefficient of variation was 7.1% at 0.15 µg/L and 5.8 % at 0.41 µg/L (n=38).

Ethical aspects

All studies were approved by the ethical research committee at the Lund University Hospital, Lund, Sweden.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS for Windows, version 14.0, Chicago, IL) and the commercial programs Stata (Ver 9) and StatXact (Ver). For specific statistical methods, see the separate papers I-VI included in this thesis.

Paper I

The area of brain biomarkers and cerebral infectious disease has not been studied in any great detail. Also, the significance of brain biomarker levels in infections outside the brain has not been established. A study was therefore designed to examine admission S100B levels in patients with CNS infections compared to patients with infectious disease outside the brain.

During the years 1995-2002, acute-phase serum samples, taken from patients seeking care for suspected infectious disease, have been frozen at the Department of Infectious Diseases at the Lund University Hospital, Lund, Sweden. A retrospective journal search was performed aiming at finding patients (age ≥ 15 years) who received the following diagnoses; bacterial meningitis (confirmed with CSF sampling including culture), bacterial pneumonia (x-ray and positive blood cultures for Streptococcus pneumoniae), viral meningitis (based on CSF analysis and clinical course but not by specific viral detection assays), cerebral abscess (radiological findings and culture results), bacterial enteritis (stool cultures positive for salmonella spp. and/or campylobacter spp.), erysipelas (clinical diagnosis), viral encephalitis (EEG, PCR) and neuroborreliosis (CSF antibody detection). 57 patients were included (15 - 84 years of age, mean 39 years). None of the patients had any evident prior history of neurological

disease and other parameters such as clinical status, laboratory tests and neuroradiological examination was recorded. Another inclusion criterion was that serum was drawn within 24 hours of arrival. The clinical outcome of patients was also graded using GOS, also based upon patient records.

Paper II

In order to further examine the clinical specificity, another study was conducted, prospectively investigating acute phase S100B levels after orthopaedic fractures without any neurological injury or disease.

The clinical setting was the emergency department at the Lund University Hospital, Lund, Sweden. 55 consecutive adult (≥ 18 years of age) patients with radiologically confirmed bone fractures were included in the study. The age of the patients in the study ranged from 19 to 92 years of age (mean 60.2 +/- 20.1 years of age). All types of fractures were included. Venous sampling for S100B was carried out within 24 hours of injury time. All patients were examined first by the orthopaedic physician on call and then examined neurologically by one of the authors (*Unden, J*) to rule out any clinically evident neurological impairment. All patients were GCS grade 15 and had no clinical neurological deficits. Patients with previous neurological disease of any sort and patients who had suspected or confirmed head trauma were excluded. Patients with multiple trauma injuries were also excluded. Radiological examinations of the brain were not performed, due to ethical reasons.

Paper III

Following the specificity work on extracerebral infectious disease and orthopaedic fractures, queries concerning the source and relevance of serum S100B in multitrauma patients emanated. The next project therefore looked at a clinical situation with substantial organ damage both in the brain and in other organs, with special focus on the process of cerebral herniation. The hypothesis was that, considering the short half-life of S100B[73], brain herniation, and hence cessation of blood flow to and from

the brain, would stop S100B sources from the brain and hence isolate extracerebral S100B. S100B levels were therefore examined frequently in a patient who progressed to cerebral herniation. The patient was later operated with organ-harvesting procedures for transplantation purposes. This gave an opportunity to measure S100B levels during extracerebral inflicted trauma in vivo with abolished cerebral contamination sources of S100B.

The study illustrates a tragic case of a car accident where the patient suffered multiple injuries, including bone fractures, lung contusions and other blunt trauma contusions to the body, as well as severe head injury (admission GCS of 4). S100B sampling was performed at admission and thereafter daily. In response to ICP instability, S100B sampling was increased to once per hour (range 30 – 180 min). Despite maximum therapy, cerebral herniation occurred. Following this, organ harvesting procedures were undertaken for transplantation. S100B sampling continued during this phase. A few CSF samples were also taken in order to confirm high intracranial S100B levels after herniation.

Paper IV

In order to examine the clinical sensitivity of S100B in head injury, particularly MHI, patients with acute epidural haematoma were evaluated with admission S100B levels. The detection of this dangerous complication to MHI is essential if a biomarker is to be successful in this setting.

Serum for analysis of S100B was drawn at or near admission. 5 patients were included. The clinical setting is the Neuro-Intensive Care Unit (NICU), Department of Neurosurgery, at the Lund University Hospital in Lund, Sweden. All 5 patients were consecutive and had received neuroimaging (CT) showing epidural haematoma. Clinical data including GCS scores were recorded. Patient characteristics are presented below:

Case 1

A 42-year-old male presenting with a 20 mm thick epidural haematoma without skull fracture after closed head injury without other extracerebral injuries. The patient was transferred to our clinic for surgical treatment.

Case 2

A previously healthy 27-year-old female involved in an automobile accident. Radiological examination showed an epidural haematoma as well as small cerebral contusions. The patient also had a comminute distal radius fracture and pulmonary contusions.

Case 3

A 77-year-old male with cardiovascular disease and prior cerebral infarctation. The patient presented with cranial fractures, both epidural and subdural haematomas as well as cerebral contusions after closed head injury. Pulmonary x-rays showed signs of pneumonia and lab analysis (Troponine T) implied a myocardial infarct.

Case 4

A 14-year-old male patient who presented at a primary care centre after head injury with unremarkable symptoms and was sent home. The following morning he could not be woken and was rushed to hospital where CT scanning showed a skull fracture with a massive epidural haematoma with signs of cerebral herniation. The patient was operated with evacuation of the haematoma approximately 20 hours after the trauma.

Case 5

The final case reports a previously healthy 17-year-old male with an epidural haematoma (see figure 1 in Paper IV for CT image) after blunt head injury. The haematoma is relatively large resulting in signs of cerebral herniation. The patient also had a fractured nasal bone.

Paper V

S100B in neurocritical care had previously been examined[117]. The data presented, however, did not conform to our observations and theories of S100B in this setting.

We therefore designed a study to examine this aspect ourselves. Patients with neurotraumatic or neurovascular diseases were therefore included in a prospective study. The primary endpoint was clinical utility possibilities; more specifically, detection of secondary neurological complications. Outcome relationships were the secondary endpoints of the study.

Initially, 82 patients were included. One patient, who was post-mortem diagnosed with Creutzfeldt-Jacobs disease, was excluded. Two patients were excluded before statistical analysis; one patient whose S100B samples had not been analysed, and one patient lost due to administrative problems. Finally, 79 patients were included in the study (42 female (53%), mean age 55 years, range 16-81 years). 29 patients (37%) had head injuries and 50 patients (63%) had CVI´s. Tables 1 and 2 shows descriptive statistics over these patients (see Paper V).

Documented parameters were age, gender, diagnosis, clinical status at and during admission, neurosurgical operation, neuroradiology, (CT, MRI, angiography), TCD, ICP, laboratory parameters including cultures and outcome. The clinical outcome was graded using GOS in conjunction with clinical follow-up visits and/or journal documentation from other hospitals. Patients were dichotomised into favourable (GOS 4-5) and unfavourable (GOS 1-3) outcome.

Secondary neurological complications were defined as CT verified new/increased ischemic/oedemic area, CT verified new/increased haemorrhage, CT-verified increase of ventricle size (hydrocephalus), clinical deterioration (prolonged (> 1 hour) new neurological deficit or worsening of GCS score by 2 steps), verified meningitis and death. Changes in ICP, TCD and lab parameters were not classed as complications, but rather as co-variables in neuromonitoring.

Paper VI

Several studies concerning stroke patients seem to indicate a role for a brain specific biomarker. However, the ability of a biomarker to differentiate ischemic stroke from haemorrhagic stroke had not been reported. These conditions should theoretically differ in biomarker levels and degree of activation of the coagulation

system. We therefore planned a prospective consecutive multi-centre study including the three most promising brain biomarkers, S100B, GFAP and NSE, with a novel marker of coagulation system activation, APC-PCI. The latter marker was chosen due to the theoretical idea that the degree of activation of the coagulation system should differ between ischemic and haemorrhagic stroke. APC-PCI has shown promising results referring to other clinical circumstances[147,148,150].

Three university hospitals in Sweden (Lund, Malmö and Umeå) participated. The study period was between April and November of 2004. In order to attain a clinically relevant and representative patient group, all patients admitted with typical symptom presentation consistent with stroke, within the last 24 hours, were included. Patients with symptoms consistent with subarachnoid haemorrhage and patients with previous stroke were excluded. Patients who, during the study period, showed clinical findings consistent with a transient ischemic attack (TIA) or other causes than stroke were also excluded.

A total of 127 patients were initially considered for inclusion. Five patients were excluded since they had other diagnoses (4 TIA and one Bell's paresis). In 16 patients, blood samples were collected after 24 hours. They were also excluded. In 9 patients, essential data was missing. Therefore, a total of 97 patients (34 females, mean 70 +/- 13 years, age range 25-95 years) formed the study population.

Serum samples for biomarker analysis were drawn on admission, before neuroimaging, (range 0.3-23.3 hours, mean 6.0 +/- 5.7 hours), centrifuged and frozen at –20 °C for later batch analysis. Serum samples were also drawn every day for 5 consecutive days. These later samples represent other study endpoints that will not be discussed further in this paper. Patients were classified according to the National Institute of Health Stroke Scale (NIHSS). All patients received CT examinations to distinguish between ischemic stroke and ICH and were also classified according to the Oxfordshire Community Stroke Project (OCSP) for subtype classification according to symptoms on admission.

Results

Paper I

Of the 57 patients that were studied, eleven patients were diagnosed with bacterial meningitis, ten with bacterial pneumonia, fourteen with viral meningitis, one patient with cerebral abscess, ten with bacterial enteritis, four with erysipelas, five with viral encephalitis and two patients with neuroborreliosis. The range of the S100B levels in the study was between 0.03 to 1.08 µg/L (mean 0.16 µg/L).

We found that 19 of the 57 patients (33%) showed elevated S100B levels (above 0.15 µg/L). Patients with viral encephalitis showed the highest mean levels of S100B in serum. Both cerebral and extracerebral infections showed individual S100B levels above normal. For graphical presentation of the results, see Paper I.

GOS was assessed between 2 weeks and 7 months after sampling (mean 3 months). The scores were GOS grade 1 (good recovery) in all cases but five. Three of these five patients had viral encephalitis (all herpes simplex virus; 0.07, 0.68 and 1.08 µg/L graded as GOS 2, 3 and 2 respectively), one had cerebral abscess (0.17 µg/L, GOS 4) and one patient had bacterial meningitis (0.26 µg/L, GOS 5 - the patient died of a ruptured aortic aneurysm two weeks after sampling, seemingly unconnected with the infection).

Paper II

The time from injury to S100B sampling was between 1 and 23 hours (mean 10.2 +/- 7.3 hours). The range of the S100B levels in the study was between 0.02 to 0.51 µg/L (mean 0.13 +/- 0.11 µg/L). We found that 16 of the 55 patients (29%) showed S100B levels above 0.15 µg/L. Fractures to larger bones tended to result in higher serum levels of S100B. Hip, tibia and radius fractures have many S100B values above the cut-off while smaller fractures to the hands and feet have values all below this limit. Patients are graphically presented, see Paper II.

Paper III

The curves of ICP and serum S100B can be seen in figure I, see Paper III later in the thesis. The first sample shows a S100B level of 2.4 µg/L that rises to 3.0 µg/L after 24 hours. On day 2, as mentioned earlier, we increased the rate of sampling to one sample per hour (range 30-180 minutes) in response to an increase in ICP and blood pressure. Cerebral herniation is assumed to have taken place at around 46 hours after admission in conjunction with the massive increase in ICP. S100B levels start to rise before this increase in ICP and peak prior to herniation and fall rapidly thereafter to a level of 0.9-1.1 µg/L. CSF analysis of S100B showed very high levels of the protein at this stage; in excess of 7000 µg/L.

Figure II (see Paper III) shows the temporal profile of S100B during extracerebral organ-harvesting procedures. The level before surgery were 1.0 µg/L which increased during the procedure to a high of 1.9 µg/L. 0-17 minutes indicate the first incision and dissection of adipose tissue down to the sternum and peritoneum. 17-94 minutes represent thoracotomy and manipulation of peritoneal organs. 94-137 minutes represent manipulation and freeing of the heart and lungs. 137-175 minutes represent continued freeing of heart and lungs and also the freeing of the ascending aorta and finally the clamping of the distal aorta under the renal vessels. At 162 minutes the patient went into atrial fibrillation. Between 175-185 minutes preparations were made for final clamping and removal of organs. The heart stopped at 190 minutes and was quickly removed followed by the removal of other organs. The final sample was achieved by forced aspiration due to the absence of circulation.

Paper IV

The five cases are presented briefly below (see also Table 1 in Paper IV).

Case 1

S100B was 0.15 µg/L taken 16 hours after the initial trauma. The patient was classed at this time to have a GCS of 13.

Case 2

S100B was measured to be 0.45 µg/L at 13 hours after the initial trauma when at GCS 11. S100B values achieved a maximum of 0.83 µg/L at 17 hours and then fell steadily to undetectable levels 5 days after trauma.

Case 3

The first S100B level measured was 0.49 µg/L, measured approximately 36 hours after initial trauma. At this time the patient was classed as GCS 10. S100B rose to a maximum of 0.68 µg/L on day 4.

Case 4

S100B was measured to be 0.20 µg/L at admission (at this time GCS 4), immediately prior to surgical intervention, a total of approximately 20 hours after the initial head trauma.

Case 5

S100B was 0.14 µg/L 3.5 hours after the initial trauma when he was GCS 11. S100B immediately postoperatively, 6 hours after trauma, was 0.10µg/L.

Paper V

Primary endpoint

Seventeen (22 %) patients suffered secondary neurological complications. CT scans revealed the complications in 8 patients (5 new/increased haemorrhages, 2 hydrocephalus and 1 patient with oedema progression), 6 patients clinically deteriorated, 2 patients died and 1 patient developed meningitis (confirmed by culture).

Mean S100B levels were found to be an independent parameter associated with secondary neurological complication ($p = 0.03$). Using a linear mixed model analysis, we found mean S100B levels to be significantly higher in patients with complications, compared to patients without, on both the day of the complication and the day afterwards ($p = 0.033$ and $p = 0.015$ respectively). There was no such difference on the day prior to the complication ($p = 0.62$). Figure 1 (see Paper V) shows time trends for serum S100B in the 17 patients on the days surrounding the complications. Despite the

observed associations, S100B measurements did not predict the occurrence of such complications. Due to this, no further analysis comparing S100B measurements with other monitoring parameters were made.

Secondary endpoint

A linear regression analysis showed age ($p = 0.003$) and admission GCS scores ($p < 0.001$), but not mean S100B ($p = 0.182$) or peak S100B ($p = 0.37$) levels, to be independent parameters associated with outcome according to dichotomised GOS. To clarify the results of S100B measurements, table 3 presents mean S100B levels with GOS scores. Due to this failure in prediction regarding S100B results, no further statistical analysis was conducted.

Paper VI

Of the 97 patients included in the study, 83 (86%) had ischemic stroke and 14 (14%) had ICH. Descriptive statistics of the biomarker levels and other variables are presented in table 1, see Paper VI.

There were no significant differences in S100B or NSE levels between ischemic stroke and ICH patients ($p = 0.13$ for S100B, $p = 0.67$ for NSE). GFAP concentrations were higher in ICH patients than in patients with ischemic stroke ($p = 0.0057$). There was no difference in APC-PCI concentration between patients with ICH and ischemic stroke ($p = 0.84$), However, almost all ICH patients had APC-PCI levels concentrated in the middle quartiles (see figure 1 in Paper VI).

In order to create a prognostic indicator for ICH we tried various cut-off levels for GFAP and APC-PCI. GFAP > 40 ng/L was the most significant prognostic variable for ICH ($p = 0.0027$). As is evident from the above remark on APC-PCI levels, a transformation of APC-PCI into an indicator variable must be of the type "neither very high nor very low". We found that having APC-PCI above 0.20 ng/L but below 0.35 ng/L was a very strong predictor for ICH ($p = 0.0004$). Further statistical information is presented in table 2 and graphs 1 and 2, see Paper VI.

To investigate the combination of GFAP and APC-PCI analysis in the prediction of ICH, one can proceed in two ways: (i) by predicting ICH when both GFAP and

APC-PCI suggest ICH (p = 0.0001); (ii) by predicting ICH when at least one of them does so (p = 0.015). The results of these combinations are seen in table 2. As the NPV is most important to satisfy our endpoint of being able to rule out ICH, we use prediction rule (ii) for further analysis.

For patients with duration of symptoms of less than 12 hours, and predicting ICH when at least one of GFAP or APC-PCI suggests ICH, we found NPV´s for ICH of 100% (3h gives p = 0.0011, 6h gives p =0.0037 and 12 h gives p =0.015 respectively, see table 2 in Paper VI).

General discussion

Almost every organ system in the body has a biomarker, or biomarker panel, to aid clinicians to make decisions. This is not the case for the brain, the most complicated and respected organ in the body. There is little doubt that a biomarker of brain injury would greatly improve the diagnostics, management and prognostics of neurological disease.

Over the last 15 years, there has been increasing evidence that S100B could function as a novel biomarker of brain damage[1,3,5,8-13,15,17,19,23,26,30,31,33-35,38-42,46,48,49,51-55,60,62,63,65,67,69,70,72,75,79,86,87,89,90,92,93,95-97,100,103,107,111,113-120,122,125,129-133,135,140,154-165]. Other markers have shown some potential, particularly GFAP, but their documentation cannot be compared to that of S100B. Many scientists in this field would argue that we are nearing clinical practice with S100B. This was even the case in the year 2002 when this thesis was initiated, especially in the management of minor head injury. However, certain aspects were not explored and concerns about the accuracy of S100B in clinical situations had been raised[4]. Particularly, worries regarding the clinical specificity of S100B sparked a lively discussion in the field[48,112,124,131]. The sensitivity of S100B has always been assumed to be very high, nearing 100%[125], but specific clinically relevant situations had not been investigated. Reports of S100B use in severe head injury and neurointensive care seemed promising, but had theoretical drawbacks and did not conform to our own unpublished observations. Concerning acute management of stroke, little work considering biomarker differences between ischemic and haemorrhagic stroke had been established.

S100B specificity

It has been known for some time that small amounts of S100B could be found in tissues outside the CNS[44,65,76,77]. However, the clinical impact of these potential sources has been unclear and previously assumed to be unimportant. In light of

recently published work[4], Papers I, II and III examine the specificity of S100B in clinically relevant situations.

All three papers clearly show clinically relevant release of S100B into serum after extracerebral tissue damage; non-cerebral infections, isolated acute non-head bone fractures and multitrauma injuries.

One can only speculate on the actual source of S100B in these patients. Theoretically, tissue damage to extracerebral areas may result in leakage of neurotoxic substances causing brain damage and S100B leakage. This explanation is, however, very unlikely as all patients were neurologically healthy and intact. Also, in Paper III, the cerebral S100B source is cut off by cerebral herniation. S100B levels do fall, but assume a stable level and then increase again in conjunction with extracerebral surgery. More likely is that damage to tissues and cells such as chondrocytes, adipose tissue and muscle constitute the contamination source. These tissues contain lower concentrations of S100B but they are greater in mass and could therefore easily result in the observed elevations. The levels are generally comparable to levels seen after uncomplicated minor head injury. This confirms the study by *Anderson et al* and also looks at patient groups more relevant to the potential patient group of a future brain biomarker. Since this, other studies have confirmed the lack of specificity of S100B[134,136].

These results limit the usefulness of S100B in predicting brain injury, i.e. the positive predictive value is compromised. An elevation of S100B in patients is therefore not necessarily diagnostic of brain injury at all. Other markers may show better specificity, such as GFAP[99,103,104]. Further studies may clarify this.

S100B Sensitivity

In order for a marker to be reliable in head injury, the clinical sensitivity must be near 100%, considering the detrimental morbidity and mortality issues of missed complications. Paper IV investigates the most feared complication of MHI, the

epidural haematoma. The findings show, in general, surprisingly low levels of S100B. One can argue that the time duration from trauma to sampling was long in many of the patients. However, these patients merely represent the clinical reality that a brain biomarker would be implicated in. Furthermore, Case 5 shows a level of S100B under the normal cut-off of 0.15 μg/L only a few hours after the trauma. The CT image presented in Paper IV demonstrates a large epidural haematoma, time-wise very near S100B sampling.

At the time of publication, this new information severely damaged the credibility of S100B since its sensitivity and negative predictive power has been assumed to be very high[125]. However, recent evidence has implicated a lower cut-off for the negative predictivity of S100B of only 0.10 μg/L[12] (and *Muller K et al* submitted 2006) in management of MHI. This evidence is furthermore based upon a large patient material. Despite this, Paper IV shows that near-normal levels of S100B after head injury should not be interpreted as "near-normal" intracranial pathology. Even slightly elevated S100B levels may indicate the presence of life-threatening intracranial complications.

S100B in infectious disease

The diagnosis of CNS infectious disease is not always simple and the diagnostic process is often time-consuming with definite diagnosis craving results from culture tests and/or other laboratory analyses. The prognosis of patients is also difficult to predict, especially in cases where brain damage is substantial. The acquisition of a reliable marker for brain injury would be welcomed in the field of infectious medicine, as both a diagnostic and prognostic tool.

In Paper I infections affecting the brain generally showed higher S100B levels than those of extracerebral nature. When comparing the subgroups of cerebral-associated infections, we found that viral encephalitis displayed the highest S100B levels (see Paper I for visual presentation). This is not surprising since these infections are known to cause cellular damage to the brain. Bacterial meningitis showed higher S100B levels than viral meningitis, which is also expected, as bacterial meningitis

affects the brain tissue to a higher extent than viral meningitis. These data seem to support the idea that S100B may function as a marker for brain damage in infectious disease.

However, some cases of brain infections showed normal levels of S100B. One could argue that these patients did not have any brain damage. However, four of these patients (one with cerebral abscess, one with bacterial meningitis and two with viral encephalitis) with normal S100B levels displayed clinical abnormalities suggesting cerebral damage, such as focal neurological deficit and decreased level of consciousness. This could be due only to a transient cerebral dysfunction. However, two of these cases also had GOS scores ≠ 5, i.e. displayed some sort of morbidity. The work is retrospective and includes few patients from each category and so conclusions are somewhat difficult to properly substantiate. A larger, prospective, study could clarify this, including multi-variant analysis. However, it does seem clear that very high S100B levels in these patients strongly suggest encephalitis. S100B in serum could therefore have a diagnostic role in the detection of this feared condition.

S100B in neurointensive care

S100B has been stipulated to be useful in the NICU setting. Our results from Paper III show that S100B might have a role in prediction of cerebral herniation, with levels peaking immediately prior to this. This observation could be confirmed with future studies.

In Paper V we examined the possible clinical utility of daily S100B determination in detection of secondary neurological complications in the NICU. Our results did not support previous conclusions[117]. Although we found associations between the S100B levels and complications, we did not see that S100B could predict these. One obvious reason for the lack of promising results is the sampling frequency. To monitor the brain in an intensive care unit with only daily tests is contradictory in terms. More frequent sampling must be evaluated.

S100B was not predictive of outcome in our study. This is rather surprising, considering the many studies showing good correlation of S100B to outcome scores[1,15,19,23,26,35,41,49,51-53,62,63,67,70,72,86,87,89,94,100,103,110,111,113-115,118-120,126,132,133,155,156,158,160-162,164,165]. However, there is an important theoretical aspect here which must be considered. Biomarkers are generally considered to be quantitative measures of tissue damage. The brain is an exceptionally qualitative organ. Minute lesions to the brain stem, for instance, can often have a much larger impact on outcome than much larger lesions to the frontal lobes. Possibly, in a heterogeneous brain damage model such as head injury, biomarkers may have a role in outcome prediction in combination with qualitative measures, such as clinical evaluation scales.

This study does not support the use of S100B in the NICU setting in this way and illuminates aspects which are important for other potential biomarkers of brain damage. It is possible that a higher sampling frequency (for instance hourly sampling) is more useful. First, however, the cost-benefit issue of such a measure should be examined. How many sampling points would be needed for one change in patient treatment? Further studies will elucidate this. Other biomarkers (GFAP has recently shown promising results[99,104]) and possibly other sampling techniques (such as sampling through microdialysis catheters[137]) are welcomed.

Stroke differentiation

Our results show that biomarkers GFAP and APC-PCI can, as independent variables or in combination, with a very high degree of reliability rule out ICH in acute stroke patients. This aspect is one of the primary goals in the acute management phase of such patients. Using the combination of GFAP and APC-PCI, a NPV of over 97% was found for ICH. Considering only patients with duration of symptoms of less than 12 hours, the NPV was 100%. Previously promising biomarkers S100B and NSE failed to show any significant difference between ICH and ischemic stroke. This may be due to the difference in biomarker profiles; these markers may show more useful results in later stages of stroke. For instance, GFAP has previously been shown to have

a quicker appearance in serum and have a different temporal profile than S100B[54]. Analysis of the markers after 24 hours is not attempted here, as the clinical application with respect to our endpoint, becomes limited after such a time period.

APC-PCI levels in ICH patients were lower and in a more narrow interval when compared to ischemic stroke patients (figure 2 in Paper VI). This might be caused by a consumption of coagulation factors during the haemorrhagic process in the brain. By contrast, ischemic stroke patients had APC-PCI concentrations within a wide range (table 1, see Paper VI). We hypothesized that smaller infarcts activates the coagulation system to a lesser extent and hence display lower APC-PCI levels. This was confirmed by comparing OCSP scores in these patients. Patients with APC-PCI levels ≤ 0.20 ng/L were compared to patients with APC-PCI levels ≥ 0.35 ng/L relating to the occurrence of smaller ischemic strokes (LACS) and larger infarcts (PACS and TACS). Using Fishers exact test, the difference was significant; $p = 0.035$.

The results of GFAP confirm recently published data[32] although our results indicate a higher cut-off level for GFAP and generally higher GFAP levels throughout. This may be due to the different GFAP assays used (*Foerch et al* used a research assay while we used a relatively established assay[127]. Also, the patients in our study were included within 24 hours of symptom onset. Although the small number of patients limits the possibility to evaluate the ability of the methods to exclude ICH, our results show that in patients with a shorter duration of symptoms, ICH could be excluded with even higher NPV. However, it is important to investigate up to 24 hours of symptom duration since therapeutic interventions, even thrombolytic agents, may well be indicated in later stages of the disease[43,84]. Our patient material is merely representative for a typical stroke population seeking care in the emergency room. A larger study including multi-variant analysis may confirm our findings.

Summary and main conclusions

- The clinical specificity of serum S100B is low. Elevated levels of S100B, in patients with clinical evidence of extracerebral tissue damage, should not be interpreted as brain damage.

- The sensitivity of serum S100B in head injury is high. However, epidural haematomas can show near-normal levels of S100B. Therefore, the magnitude of S100B levels in head trauma patients should not be directly related to the risk and/or magnitude of intracranial pathology.

- Serum S100B is generally elevated in CNS infections, especially in encephalitis where levels were found to be high.

- Most of the circulating S100B in serum after multitrauma injuries originates from the brain.

- Daily serum S100B levels are not clinically useful in the neurointensive care setting.

- Serum GFAP and APC-PCI measurements, prior to neuroimaging, can accurately rule out haemorrhagic stroke in a representative stroke population.

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