Surgically Treated Intracerebral Haemorrhage

Pathophysiology and
Clinical Aspects

Lovisa Tobieson

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To Ester!

If it doesn't challenge you it doesn't change you!
/the inside of my Ironman cap 😊

ABSTRACT

Mortality and morbidity of intracerebral haemorrhage (ICH) is excessively high, and the case fatality rate has not improved in the last decades. Although surgery for ICH can be life-saving, no positive effect on functional outcome has been found in large cohorts of ICH patients. Increased understanding of the pathophysiology of ICH is needed to develop improved treatment strategies.

In 17 ICH patients, paired cerebral microdialysis (CMD) catheters were inserted in the perihemorrhagic zone (PHZ) and in normal uninjured cortex at time of surgery. Despite normalisation of cerebral blood flow, a persistent metabolic crisis indicative of mitochondrial dysfunction was detected in the PHZ. This metabolic pattern was not observed in the uninjured cortex.

CMD was also used to sample proteins for proteomic analysis. A distinct proteome profile that changed over time was found in the PHZ when compared to the seemingly normal, uninjured cortex. However, protein adsorption to CMD membranes, which may interfere with concentration measurements, was substantial.

Surgical treatment of 578 ICH patients was analysed in a nation-wide retrospective multi-centre study in Sweden over five years. Patients selected for surgery had similar age, pre-operative level of consciousness and co-morbidity profiles, but ICH volume and the proportion of deep-seated ICH differed among the six neurosurgical centres. Furthermore, there was variability in the post-operative care, including the use and duration of intracranial pressure monitoring, cerebrospinal fluid drainage and mechanical ventilation.

In conclusion, the results of this thesis show that:

- (i) Despite surgical removal of an ICH a metabolic crisis caused by mitochondrial dysfunction, a potential future therapeutic target, persists in the perihemorrhagic zone.
- (ii-iii) CMD is a valuable tool in ICH research for sampling novel biomarkers using proteomics, which may aid in the development of improved therapeutic interventions. However, caveats of the technique, such as protein adsorption to the CMD membrane, must be considered.
- (iv) The nation-wide study illustrates similar clinical features in patients selected for ICH surgery, but substantial variability in ICH volume and location as well as neurocritical care strategies among Swedish neurosurgical centres. Development of refined clinical guidelines may reduce such inter-centre variability and lead to improved functional outcome for ICH patients.

KIRURGISK BEHANDLING AV HJÄRN- BLÖDNINGAR

Spontan primär hjärnblödning (ICH), som inte beror på pulsåderbräck, utgör ca 12.5% av alla strokeinsjuknanden per år i Sverige, och står för en ansevärd del av den årliga stroke-relaterade dödligheten och förlusten av funktion. Endast en liten del av patienterna kan rehabiliteras åter till ett självständigt liv. I dagsläget saknas effektiv behandling utöver att förebygga att blödningen ökar i storlek genom snabb korrigering av koagulationsrubbningar och blodtryckssänkande behandling. Ca 5 % av alla ICH i Sverige opereras, men det saknas tydlig evidens för nyttan av kirurgi utöver som livräddande åtgärd.

I dagsläget är sjukdomsmekanismerna vid ICH ofullständigt kända. Den grundläggande hypotesen är att sekundära skadefaktorer, som leder till förvärrad hjärnskada, fortgår efter att blödningen orsakat en primär skada. Dessa sekundära skadefaktorer kan kvarstå i randzonen kring ett ICH trots kirurgisk utrymning av blödningen. Ökad kunskap om hjärnskadeutvecklingen efter ICH är nödvändig för att utveckla nya behandlingsmetoder. Detta kan exempelvis uppnås genom mikrodialys; en metod som används rutinmässigt inom neurointensivvården för neurokemisk monitorering.

Mikrodialys innebär att en tunn kateter, med ett poröst semipermeabelt membran i änden, läggs i hjärnvävnaden. Katetern genomspolas av en vätska, s.k. perfusat, med en konstant hastighet. Molekyler diffunderar från hjärnvävnaden, över membranet, till perfusativätskan, och följer sen med denna vätska ut genom katetern och samlas upp som dialysativätska.

I denna avhandling användes mikrodialys i randzonen kring en utrymd blödning, samt i mer normal och oskadad vävnad på visst avstånd från blödningen för jämförelse. Vid analys av dialysativätskan från randzonen fann vi ett metabolt mönster som indikerade en energistörning orsakad av mitokondriell dysfunktion, dvs. att de celldelar som producerar merparten av cellens energi har nedsatt funktion. Genom att kombinera mikrodialys med kartläggning av blodflödet runt den utrymda blödningen kunde vi visa att energistörningen kvarstår trots att blodflödet återhämtat sig. Mitokondriell dysfunktion kan vara en viktig sekundär skademekanism efter ICH, och en möjlig angreppspunkt för framtida behandling.

Vidare studerades skillnader i proteinuttryck, s.k. proteomik, i vävnaden nära blödningen jämfört med i oskadad vävnad. Flera proteiner som tidigare har visats ha nervcellsskyddande egenskaper i andra sjukdomar eller djurmodeller uttrycktes i ökande grad både i vävnaden nära den utrymda blödningen (haptoglobin, transthyretin) men också i normal hjärnvävnad (dermcidin). Resultaten antyder att det finns potentiellt skyddande faktorer vars uttryck i hjärnan ökar efter en blödning. Möjligen kan dessa kroppsegna skyddsmekanismer visa sig användbara som del i framtida behandlingar. Vidare påvisades att proteiner till en betydande del fastnar på mikrodialysmembranen, och därmed inte alls eller endast i liten mängd passerar över till dialysatvätskan, vilket påverkar analysresultaten. Detta poängterar vikten av att studera proteiner som fastnat på membranet när mikrodialys används i kombination med proteomik.

I en retrospektiv multicenterstudie kartlades samtliga patienter som opererats för hjärnblödning på de sex neurokirurgiska klinikerna i Sverige under en femårsperiod, och indikationer för operation och behandlingsresultat jämfördes. Behandlingsstrategierna varierade mellan klinikerna med avseende på hur länge patienterna behandlades i respirator, i vilken utsträckning tryckmätare opererades in i hjärnvävnaden, och hur dränering av likvor användes efter kirurgi. Det fanns en samstämmighet mellan klinikerna avseende vilka patienter som erbjudits kirurgi, gällande exempelvis ålder och medvetandegrad, men även en del skillnader där en klinik opererade blödningar av mindre volym, och en annan fler djupt liggande blödningar. Det förelåg endast små skillnader i 30-dagars mortalitet, och den befanns vara i linje med, eller lägre än, aktuella internationella resultat.

Sammanfattningsvis har arbetena som ingår i denna avhandling visat följande:

- (i) Trots att en hjärnblödning utryms kirurgiskt så kvarstår en metabol kris, orsakad av mitokondriell dysfunktion, i randzonen kring blödningen. Denna kan vara en angreppspunkt för nya behandlingar för ICH.
- (ii-iii) Mikrodialys är en användbar metod för att studera hjärnskada efter ICH och kan kombineras med proteomik analys men det finns fallgropar och svårigheter med tekniken att ta hänsyn till, såsom att proteiner fastnar på mikrodialysmembranet.
- (iv) Den nationsövergripande genomgången av kirurgiskt behandlade ICH patienter påvisar likheter avseende patienternas ålder och medvetandegrad, men skillnader i volym och lokalisation av opererade blödningar, samt i den postoperativa vården. Dessa skillnader bör vara fokus för framtida forskningsstudier för att möjliggöra utformningen av bättre riktlinjer för klinisk praxis.

CONTENTS

ABSTRACT.....	I
KIRURGISK BEHANDLING AV HJÄRNBLÖDNINGAR.....	III
CONTENTS.....	V
LIST OF FIGURES	VII
LIST OF TABLES.....	VIII
LIST OF PAPERS.....	IX
ABBREVIATIONS	XI
ACKNOWLEDGEMENTS.....	XII
1 INTRODUCTION.....	1
2 BACKGROUND.....	3
2.1 Epidemiology.....	3
2.1.1 Risk factors.....	3
2.1.2 Causes.....	4
2.1.3 Prognostic factors.....	6
2.1.4 Long-term outcome	7
2.2 Pathophysiology.....	7
2.2.1 Increased intracranial pressure	8
2.2.2 Oxidative stress.....	11
2.2.3 Inflammation	11
2.2.4 Mitochondrial dysfunction.....	12
2.3 Current treatment guidelines	14
2.3.1 Clinical presentation and diagnosis.....	14
2.3.2 Medical treatment	15
2.3.3 Surgical treatment.....	17
2.4 Neurocritical care of ICH patients	22
2.4.1 Cerebral blood flow	22
2.4.2 Cerebral microdialysis	23
2.4.3 Cerebral microdialysis for sampling macromolecules	29
3 AIMS.....	33
4 METHODS.....	35
4.1 Patients	35
4.1.1 Inclusion and exclusion criteria (Papers I-IV)	35
4.1.2 Data collection (Papers I-IV).....	36
4.1.3 Neurocritical care protocol (Paper I-III).....	37
4.1.4 Surgical procedures (Paper I-III).....	37
4.1.5 Handling of samples (Paper I-III).....	39
4.2 Cerebral blood flow (Paper I)	40
4.2.1 Xenon-enhanced computed tomography.....	40
4.2.2 Perfusion computed tomography	42
4.3 Microdialysis (Paper I-III).....	42
4.3.1 Interpretation of low-molecular-weight metabolites	43
4.4 Proteomics (Paper II & III).....	43
4.4.1 Sample preparation.....	46
4.4.2 2-DE-based proteomics	46
4.4.3 MALDI-TOF-MS	48
4.4.4 LC-based proteomics	49
4.5 Statistical methods.....	52

4.5.1	<i>Non-parametric tests</i>	52
4.5.2	<i>Parametric tests</i>	52
4.5.3	<i>Multilevel mixed linear model (Paper I-II)</i>	53
4.5.4	<i>OPLS-DA for –omics analysis (Paper II)</i>	54
5	RESULTS	57
5.1	Patients (Paper I-III)	57
5.2	Cerebral blood flow (Paper I)	58
5.3	Microdialysis (Paper I & II)	59
5.4	Proteomics (Paper II & III)	62
5.4.1	<i>Proteome of microdialysate (Paper II)</i>	63
5.4.2	<i>Proteome of membranes (Paper III)</i>	66
5.5	Neurocritical care and surgery for ICH in Sweden (Paper IV)	67
6	DISCUSSION	73
6.1	Metabolic crisis in perihemorrhagic zone (Paper I)	73
6.2	Proteomic analysis of microdialysate and membranes (Paper II & III)	75
6.3	Neurocritical care and surgery for ICH in Sweden (Paper IV)	80
6.4	Limitations	81
6.4.1	<i>Patient inclusion</i>	81
6.4.2	<i>Data collection</i>	82
6.4.3	<i>Microdialysis</i>	83
6.4.4	<i>2-DE Proteomics</i>	84
6.4.5	<i>LC-based proteomics</i>	85
7	CONCLUSION	87
8	FUTURE DIRECTIONS	89
	REFERENCES	91
	INDEX	107

LIST OF FIGURES

Figure 2.1 Deep-seated versus lobar ICH.	4
Figure 2.2 Secondary brain injury of ICH	9
Figure 2.3 Perihaemorrhagic edema (PHE).....	10
Figure 2.4 Electron transport chain	12
Figure 2.5 Mitochondrial permeability transition pore (MPTP).	13
Figure 2.6 Basic principle of cerebral microdialysis (CMD).	23
Figure 2.7 Tissue factors influence relative recovery (RR).....	25
Figure 2.8 Overview of ATP production.	28
Figure 4.1 Pre- and post-operative CT scan	39
Figure 4.2 Xe-CT evaluation of CBF.	41
Figure 4.3 Gel-based versus LC-based proteomics.	45
Figure 4.4 LC-based proteomics approach.	50
Figure 4.5 Hierarchical data	53
Figure 4.6 Multivariate analysis workflow.....	55
Figure 5.1 CBF heat-map.....	59
Figure 5.2 CMD low-molecular-weight metabolites.	60
Figure 5.3 Proportion of type 1 versus 2 LPR elevations.	62
Figure 5.4 Typical 2-DE gel of perihaemorrhagic microdialysate.	64
Figure 5.5 OPLS-DA score plots.	65
Figure 5.6 Differences in proteome profile.	66
Figure 5.7 Frequency distribution of age.....	68
Figure 5.8 Volume and proportion of deep-seated ICH.	70
Figure 6.1 Protein increase mechanisms	77
Figure 6.2 Electron micrographs of perfused tissue	78
Figure 6.3 Protein inference problem.	85

LIST OF TABLES

Table 2.1. Main causes of ICH.....	5
Table 2.2 Normal and pathological levels of routinely measured low-molecular-weight metabolites.	27
Table 4.1 Translation RLS-85 to GCS-M.	37
Table 5.1 Proportion of type 1 versus type 2 LPR elevation in PHZ and SNX at early and late time period.	61
Table 5.2 Incidence of surgery for ICH per 100,000 person-years	67
Table 5.3 Descriptive statistics incl. missing values.	69
Table 5.4 Neurocritical post-operative care strategies.	71

LIST OF PAPERS

- I. **Tobieson L**, Rossitti S, Zsigmond P, Hillman J, Marklund N. Persistent metabolic disturbance despite a normalized cerebral blood flow in the perihemorrhagic zone following surgery for intracerebral hemorrhage. *Neurosurgery*. 2018 May 21. doi: 10.1093/neuros/nyy179. [Epub ahead of print].
- II. **Tobieson L**, Ghafouri B, Zsigmond P, Rossitti S, Hillman J, Marklund N. Dynamic protein changes in the perihemorrhagic zone of surgically treated intracerebral hemorrhage patients. *Scientific Reports*. 2019 Feb 28;9(1):3181. doi: 10.1038/s41598-019-39499-2.
- III. **Tobieson L**, Czifra Z, Wåhlen K, Marklund N, Ghafouri B. A proteomic investigation of protein adsorption to cerebral microdialysis membranes in surgically treated intracerebral hemorrhage patients. (submitted)
- IV. Fahlström A*, **Tobieson L***, Redebrandt H, Zeberg H, Bartek J Jr, Bartley A, Erkki M, Hessington A, Troberg E, Mirza S, Tsitsopoulos PP, Marklund N. Differences in neurosurgical treatment of intracerebral haemorrhage: a nation-wide observational study of 578 consecutive patients. *Acta Neurochirurgica* (Wien) 2019 Mar 15. doi: 10.1007/s00701-019-03853-0. [Epub ahead of print]

* Contributed equally.

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Related papers not included in PhD:

- Tsitsopoulos PP, **Tobieson L**, Enblad P, Marklund N. Prognostic factors and long-term outcome following surgical treatment of 76 patients with spontaneous cerebellar haematoma. *Acta Neurochirurgica* (Wien). 2012 Jul; 154(7):1189-95. doi: 10.1007/s00701-012-1372-7. Epub 2012 May 18.
- Tsitsopoulos PP, Enblad P, Wanhainen A, **Tobieson L**, Hårdemark HG, Marklund N. Improved outcome of patients with severe thalamic hemorrhage treated with cerebrospinal fluid drainage and neurocritical care during 1990-1994 and 2005-2009. *Acta Neurochirurgica* (Wien). 2013 Nov;155(11):2105-13. doi: 10.1007/s00701-013-1871-1. Epub 2013 Sep 13.
- Fahlström A, Nittby Redebrandt H, Zeberg H, Bartek Jr J, Bartley A, **Tobieson L**, Erkki M, Hessington A, Troberg E, Mirza S, Tsitsopoulos PP, Marklund N. The Surgical Swedish ICH Score - A Grading Scale for Surgically Treated Patients with Spontaneous Supratentorial ICH. (submitted)

ABBREVIATIONS

2-DE	Two-dimensional gel electrophoresis
BBB	Blood-brain-barrier
CBF	Cerebral blood flow
CMD	Cerebral microdialysis
CSF	Cerebrospinal fluid
CTP	Computed tomography perfusion
EVD	External ventricular drain
FDR	False discovery rate
GCS	Glasgow coma scale
ICH	Intracerebral haemorrhage
ICP	Intracranial pressure
LC	Liquid chromatography
LPR	Lactate/pyruvate ratio
MD	Microdialysis
MML	Multilevel mixed linear
MPTP	Mitochondrial permeability transition pore
MS/MS	tandem mass spectrometry
NCC	Neurocritical care
OPLS-DA	Orthogonal projection to latent structures discriminant analysis
PHE	Perihaemorrhagic edema
PHZ	Perihaemorrhagic zone
RLS-85	Reaction level scale
ROI	Region of interest
ROS	Reactive oxygen species
SAH	Subarachnoid haemorrhage
SNX	Seemingly normal cortex
TBI	Traumatic brain injury
VIP	Variable of importance in projection
Xe-CT	Xenon-enhanced computed tomography

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Now – onwards and upwards!

Linköping
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1 INTRODUCTION

Spontaneous supratentorial intracerebral haemorrhage (ICH) is a devastating disease with excessively high mortality and morbidity rates (1, 2). In Sweden approximately 23,000 patients suffer supratentorial ICH every year and the functional outcome is unfavourable for a large proportion of these patients despite best medical and surgical treatment (3). The reasons for the poor treatment results have not yet been clarified. A better knowledge of the pathophysiology and secondary brain injury processes following ICH can lead to development of more successful interventions and thereby improve functional outcome for patients.

In contrast to an ischemic stroke which has a hypoxic but salvageable penumbra of tissue surrounding an infarcted core, the ICH is not surrounded by an ischemic penumbra but rather by a biochemical one. This biochemical penumbra is characterised by a metabolic crisis (4-6) in the tissue surrounding an ICH (the perihemorrhagic zone; PHZ) not explained by impaired local cerebral blood flow (CBF) and hypoxia (7). Instead dysfunctional or damaged mitochondria have been implicated. Such mitochondrial dysfunction could be an important part of the pathophysiology of ICH but has only been sporadically indicated in previous studies of ICH (5, 6). Mitochondrial dysfunction does, however, represent an area of growing research for both acute brain injury (8-10) and neurodegenerative diseases (11-14), and is a possible target for future therapies. In this thesis cerebral microdialysis (CMD) combined with measurements of regional CBF was used to investigate to what extent a metabolic crisis suggestive of mitochondrial dysfunction is present in the PHZ.

Apart from monitoring the metabolic state of the brain tissue (15, 16), CMD can also be used to sample proteins and peptides (17-19). Mapping and comparing the proteins expressed in tissue surrounding the ICH and more remote areas, as done in Paper II, can improve our knowledge of ICH pathophysiology. However, there are methodological issues to consider when sampling proteins with CMD, such as protein adsorption to the membrane (19, 20), explored in Paper III.

Finally, the current lack of clinical guidelines for the surgical and post-operative care of ICH patients can lead to treatment variability which may contribute to variations in outcome. In Paper IV of this thesis such variability in Sweden was explored. In essence, the thesis aims to analyse current surgical treatment of ICH patients, and to add to the knowledge of ICH pathophysiology so that refined treatments leading to functional improvements for ICH patients can be developed.

2 BACKGROUND

This section contains a literature review summarising the epidemiology, pathophysiology, and current treatment guidelines for ICH, including the evidence for surgical treatment. The final part covers neurocritical care (NCC) of ICH patients including evaluation of cerebral blood flow, and monitoring of metabolism and biomarker investigations using cerebral microdialysis (CMD).

2.1 Epidemiology

Supratentorial spontaneous intracerebral haemorrhage (ICH) is a devastating disease with a 30-day mortality rate of 25-48% (1) and a 12-month mortality rate of >50% (21). Although the incidence of ischemic stroke is higher, the major part of the global burden of stroke measured by disability-adjusted life years (DALYs) is attributed to ICH (22), and <40% of ICH survivors regain functional independence (21). Case fatality remains high world-wide and, more worryingly, has not improved in the last decades (21). World-wide ICH incidence is 24.6 per 100,000 person-years (21) although this number varies with age, sex, and geographic region (23). Furthermore, the incidence of ICH in adults <65 years has increased by 25% in the last two decades (24), and with the increased use of anticoagulant therapy it is likely to increase also in the population >65 years. The supratentorial ICH incidence in Sweden is currently, according to the Swedish Stroke Register (Riksstroke) which has a 90% coverage, 22.9 per 100,000 person-years (3).

2.1.1 Risk factors

Hypertension, leading to vasculopathy of deep perforating arterioles, remains the most important risk factor for ICH in both young and elderly patients (25). In elderly patients, hypertension is followed by cerebral amyloid angiopathy (CAA) as the second strongest risk factor for ICH. CAA is characterised by deposition of amyloid- β peptides in the cortical and leptomeningeal vessel walls including capillaries, arterioles and small and medium size arteries (26) and leads primarily to lobar ICH (27).

Advanced age is in itself a risk factor for ICH (28), as is the use of anticoagulants, particularly in the elderly, where the incidence of anticoagulant-related ICH is increasing steadily (3). Risk factors specifically important in young patients (18-50

years) include hypertension, diabetes, menopause, current cigarette smoking, high alcohol intake, high caffeine intake, and cocaine use (23).

Modifiable risk factors for ICH include high alcohol intake, high waist-to-hip-ratio, diabetes, and current smoking. A healthy diet and regular and strenuous physical activity >4 hours per week are both protective factors (29).

2.1.2 Causes

ICH patients are heterogeneous, and the causative vascular factors such as small vessel disease, CAA, vasculitis, vascular malformations or other occult causes may go undetected unless a thorough search for the underlying cause is conducted (30). Nevertheless, approximately 80% of ICH are caused by vasculopathy of deep perforating arterioles (arteriosclerosis), causing mainly deep-seated ICH, and CAA, (30) leading to predominantly lobar ICH (Figure 2.1).

Figure 2.1 Deep-seated versus lobar ICH.

CT-scans of (a) a 69-year-old male with a deep-seated ICH and (b) a 72-year-old male with a lobar ICH.

ICH caused by hypertensive vasculopathy of deep perforating arterioles is most often located in the basal ganglia, thalamus or brainstem. As this vasculopathy has two modes of expression (occlusive and haemorrhagic) other signs of chronic arteriosclerotic brain damage, such as cerebral white matter lesions, lacunes, microbleeds or previous ICH in basal ganglia or brainstem may also be present and can support the diagnosis. In the absence of such markers of arteriosclerosis an expanded search for the underlying vascular cause should be considered (see Table 2.1) even in the elderly hypertensive patient (30).

Table 2.1. Main causes of ICH.Adopted from Cordonnier *et al.*, 2018 (30).

Cause of ICH		Possible signs indicating cause
80% of ICH	Vasculopathy of deep perforating arterioles (arterio-sclerosis)	Deep-seated ICH; microbleeds or previous ICH in basal ganglia/brain stem; white matter lesions; lacunes.
	Cerebral amyloid angiopathy (CAA)	Lobar ICH; cortico-subcortical micro-bleeds; cSS; ApoE ϵ 4; cognitive decline; transient focal neurological episodes; age >55 years.
20% of ICH	AVM	ICH extension to other brain compartments; flow voids; calcification
	Arterial aneurysm	Disproportionate cisternal subarachnoid extension of ICH
	Cavernous malformation	Small, homogenous ICH with no extension to other brain compartments
	Venous thrombosis	Headache preceding ICH onset; ICH close to sinuses or veins; high relative edema volume; ICH onset in pregnancy or postpartum; oral contraceptives.
	Dural AV fistula	Subarachnoid or subdural extension; abnormal dilated cortical vessels
	Haemorrhagic transformation of cerebral infarction	Acute ischaemic lesions adjacent to ICH or diffuse acute ischemic lesions in other arterial territories
	Coagulopathy	Abnormal coagulation tests
	Tumour (primary or secondary)	Extensive PHE
	Vasculitis	Headaches; small acute ischemic lesions in different arterial territories; focal diffuse arterial stenosis
	Infective endocarditis	Acute ischaemic lesions in different arterial territories; small irregular arterial aneurysms; diffuse brain microbleeds
	PRES	Thunderclap headaches; parietal and occipital asymmetrical edematous lesions
Abbreviations: ApoE = apolipoprotein E; cSS = cortical superficial siderosis; AVM = arteriovenous malformation; AV = arteriovenous; PHE = perihemorrhagic edema ; PRES = Posterior reversible encephalopathy syndrome		

2.1.3 Prognostic factors

Factors associated with poor functional outcome include advanced age, depressed level of consciousness at admission, diabetes mellitus (31), and pre-ICH cognitive impairment (28). In addition, >20% of ICH patients will deteriorate ≥ 2 Glasgow Coma Scale (GCS) points between the initial assessment and arrival at the emergency department, and such deterioration is associated with increased mortality and need for surgical intervention (32).

Coagulopathy and a sustained high systolic blood pressure (SBP) create an increased risk of ongoing bleeding, and are both associated with neurological deterioration (33). The risk of poor outcome following ICH is increased for all different anticoagulants in current use (28), although NOAC-treated ICH patients have smaller ICH volume, are less prone to haematoma expansion and have lower mortality than Warfarin-treated ICH patients (34-40). Approximately 25% of ICH patients are on antiplatelet therapy and their prognosis is worse than that of patients not on antiplatelet therapy (41).

The incidence of seizures in ICH is high (15-20%), the majority of which are non-convulsive (42), however the effect of seizures on patient outcome is uncertain (28). Continuous EEG monitoring is recommended to rule out seizures in patients with depressed consciousness following ICH (43, 44). Fever (45), hyperglycaemia, neutrophil-lymphocyte ratio, and serum fibrinogen level are also negative prognostic factors following ICH (28). In addition, do-not-resuscitate (DNR) orders and early decisions to withdraw life sustaining treatment are both independent negative prognostic factors in ICH (46, 47).

Strong negative predictive factors on neuroimaging include ICH location (deep-seated and posterior fossa), volume (48) and haematoma expansion (49). Spot-sign, indicated by contrast extravasation on CT-angiography, is associated to haematoma expansion and is a negative prognostic factor (50), as is PHE, intraventricular haemorrhage (IVH), and hydrocephalus (28).

Presence of poor prognostic factors may skew early treatment decisions and limit the level of care given to treatable patients generating self-fulfilling prophecies of poor prognosis. This was illustrated in a prospective study where DNR orders were postponed five days in poor grade ICH patients resulting in lower 30-day mortality than predicted by the ICH-score for all score levels (47). The guidelines suggest *initial* aggressive treatment of *all* ICH patients regardless of prognostic factors (43).

2.1.4 Long-term outcome

Long-term follow up studies in ICH patients are scarce. A small number of studies present 12-month results, and there are a few studies reporting longer observation periods (31, 51-54). One recently published study of functional outcome at five years following either conservative or surgical treatment for ICH in Sweden showed that 79% of patients had a poor functional outcome (31). The majority of studies, however, report outcome at 3-6 months post ICH.

Several ways of measuring outcome after ICH exist, all of which have their strengths and limitations. The 30-day mortality gives reliable information on the case fatality of ICH and is easily accessed in patient records. Functional outcome can be measured by different scales (55), of which the modified Rankin Scale (mRS) is the most commonly used in stroke research (56). The mRS has been criticised for low interrater reliability (57) and inadequate assessment of neuropsychological deficits. Furthermore, current stroke research places an increasing emphasis on patient reported outcome measures (PROM) exemplified by measures of quality of life (QoL) (58) such as EQ-5D (59) and Neuro-QoL (60). Such parameters are lacking in the mRS. Long-term follow up studies of functional outcome and reported QoL should be a focus for future investigations in ICH.

Despite current best medical and surgical treatment the mortality and morbidity of ICH remains high and novel treatments, such as neuroprotective therapies, are lacking. This will potentially change as knowledge accumulates giving better insight into the highly complex ICH pathophysiology and secondary injury processes it triggers.

2.2 Pathophysiology

Knowledge of ICH pathophysiology is based mainly on animal studies (61, 62), most commonly of intrastriatal injection of autologous blood in rodents. A few clinical studies using either non-invasive neuroimaging, invasive techniques such as microdialysis or biopsy, or post-mortem histopathology have also contributed to our current knowledge of ICH injury mechanisms.

Brain injury following ICH can be divided into primary and secondary processes. The primary brain injury occurs immediately at the ICH onset, involves bleeding into brain parenchyma as a consequence of vessel rupture, and leads to disruption of axons as well as glial and neuronal cells (63). Primary brain injury can only be addressed by preventive interventions targeting the modifiable risk factors of ICH.

Ongoing secondary brain injury triggered by ICH is a highly complex set of interconnected events (see Figure 2.2). Several major processes contribute to the ongoing injury including:

- raised intracranial pressure (ICP) due to mass effect of the ICH, perihemorrhagic edema (PHE) formation or acute hydrocephalus,
- oxidative stress, and
- inflammation.

These processes are the target of stroke care protocols and neurosurgical interventions, and several preclinical trials of neuroprotective drugs (64, 65).

2.2.1 Increased intracranial pressure

Increased ICP caused by haematoma expansion (see section 2.3.2.1 on page 15), acute hydrocephalus or PHE formation, can compromise cerebral perfusion pressure (CPP) and lead to a decreased cerebral blood flow (CBF). Haematoma expansion takes place in approximately one third of ICH cases (49), is more prevalent and severe in patients on anticoagulant or antiplatelet therapy (66), and is associated with worse clinical outcome (49). Interventions to limit haematoma expansion include lowering systolic blood pressure (SBP), and reversing any anticoagulant therapy (43, 44). Such medical interventions will be covered in section 2.3.2 on page 15.

In approximately 40% of cases the ICH ruptures into the ventricles causing IVH (3), which potentially leads to a disruption of normal circulation of cerebrospinal fluid (CSF), acute hydrocephalus and a subsequent rise in ICP. Increased ICP caused by mass effect can be alleviated by surgical evacuation of the ICH, or by performing a decompressive hemicraniectomy which allows the brain to expand, whereas IVH and hydrocephalus can be treated by an external ventricular drain (EVD). Such surgical treatment strategies will be covered in 2.3.3 on page 17.

Figure 2.2 Secondary brain injury of ICH

Complex interconnected cascades are triggered by the ICH. ROS = reactive oxygen species; DIC = disseminated intravascular coagulation; BBB = blood-brain-barrier; PAI-1 = plasminogen activator inhibitor 1; MMP = matrix metalloproteinase.

The ICH causes a break-down of the blood-brain-barrier (BBB) contributing to PHE (Figure 2.3), which potentially increases ICP, is associated with neurologic deterioration (67, 68) and plays a crucial role in the pathophysiology of ICH.

Figure 2.3 Perihaemorrhagic edema (PHE).

Right sided ICH in basal ganglia of 65-year-old male patient prior to surgery. The ICH is surrounded by a rather discrete perihaemorrhagic edema (PHE) indicated by black arrows.

The natural progression of PHE can be described by three phases:

(i) the *ionic edema* (within hours) characterised by intact BBB, net influx of sodium and accumulation of serum proteins in the intersitium which creates an osmotic gradient for water (69);

(ii) *vasogenic edema* (days), characterised by breakdown of the BBB and subsequent leakage of plasma proteins into the extracellular space (70, 71); and

(iii) *delayed vasogenic edema* (days to weeks) mainly mediated by haemoglobin degradation products such as haem and iron, which induce reactive oxygen species (ROS) and further breakdown of the BBB (64, 72-74).

Numerous treatment strategies aimed at reducing the PHE have been explored including the use of osmolar therapy such as mannitol or hypertonic saline, corticosteroid treatment, therapeutic hypothermia, and neuroprotective agents (70, 75). Some of these have been shown to reduce the PHE, although none have yet translated to improved functional outcome (see 2.3.2.4).

2.2.2 Oxidative stress

Blood breakdown products, in particular haem and iron, induce cytotoxic ROS (76), that cause lipid peroxidation, leading to cell membrane damage and subsequent Ca^{2+} influx in neurons and glia (77). The increase in intracellular Ca^{2+} causes activation of apoptotic pathways and thereby contributes to further ROS production. ROS also cause damage to proteins and nucleic acids, which critically alters cellular functions (78).

There are several naturally occurring protective pathways in the brain, guarding against the oxidative stress caused by blood toxicity, but these pathways get exhausted by ICH. Examples of such pathways include astrocytic release of haptoglobin (79-83) and haemopexin which scavenge haem and facilitate subsequent macrophage phagocytosis of these haem-scavenger complexes (79, 82).

2.2.3 Inflammation

The inflammatory response is a natural defence to restore tissue homeostasis following ICH. Resident microglia and astrocytes respond promptly to an ICH (84) as the extravasated blood products, particularly thrombin, activate resident microglia within minutes of ICH onset (85). Activated microglia inhibit oxidative phosphorylation, cause accumulation of pyruvate in the cytosol and extracellular fluid, and release pro-inflammatory cytokines triggering further neuroinflammatory cascades (84).

Blood-derived infiltration of immune cells occurs several days after ICH onset (86) and ongoing neuroinflammation trigger apoptotic pathways contributing to the loss of neuronal and glial cells (87). Danger associated molecular patterns (DAMP) released from injured and dead cells propagate ongoing inflammation in the latter stages of ICH induced injury (84).

Although knowledge of ICH pathophysiology has improved over the last decades, additional studies are needed (88) in order to develop better treatment strategies (88). In particular, several of the secondary brain injury processes are closely related to mitochondrial dysfunction, a topic under increasing investigation both within acute and chronic neuropathophysiology. Although only sporadically described previously in ICH (5, 6), mitochondrial dysfunction could be highly relevant in ICH pathophysiology.

2.2.4 Mitochondrial dysfunction

The mitochondria are bilayer organelles present in every cell in the body apart from red blood cells. They supply 90% of the ATP needed (89) (Figure 2.4), and apart from providing energy, the mitochondria also generate and regulate ROS, buffer cytosolic Ca^{2+} , and regulate apoptosis and necrosis pathways (89, 90).

Figure 2.4 Electron transport chain

The electron transport chain is where the oxidative phosphorylation occurs, generating the vast majority of adenosine triphosphate (ATP) molecules. Electrons (e^-) are transferred from NADH to oxygen by a series of large protein complexes in the inner membrane of the mitochondria; the respiratory chain complexes (numbered by Roman numerals) (91). This creates a transmembrane electrochemical gradient as protons (H^+) are pumped across the membrane. The flow of protons back across the ATP synthase leads to the formation of ATP. Q = ubiquinone; CytC = cytochrome C; NAD/NADH = Nicotinamide adenine dinucleotide; FADH/ H_2 = flavin adenine dinucleotide; ADP = adenosine diphosphate.

Mitochondrial dysfunction observed in acute brain injury has been linked to an improper opening of the mitochondrial permeability transition pore (MPTP) (92), a non-specific voltage-dependent protein complex spanning the mitochondrial outer

membrane and controlling the mitochondrial permeability (93). Transient ischemia/hypoxia or glutamate mediated excitotoxicity cause a disturbed Ca^{2+} homeostasis with an increase in cytosolic and mitochondrial Ca^{2+} (77). This leads to opening of the MPTP which causes both dissemination of the transmembrane electrochemical gradient with resulting failure of ATP production but also osmotic swelling of the mitochondria and subsequent rupture of the outer membrane which leads to increased release of ROS and apoptotic mediators (Figure 2.5).

Figure 2.5 Mitochondrial permeability transition pore (MPTP).

Role of mitochondrial permeability transition pore (MPTP) in mitochondrial dysfunction. ATP = adenosine triphosphate; OMM = outer mitochondrial membrane; ROS = reactive oxygen species; MRC = mitochondrial respiratory chain.

The impaired Ca^{2+} homeostasis causes an increase in catabolic intracellular processes, overproduction of reactive oxygen and nitrogen species, activation of apoptosis pathways and up-regulation of inflammatory mediators (77). The oxida-

tive phosphorylation is further disturbed by precipitation of calcium and phosphate into insoluble calcium phosphate in the inner matrix of the mitochondrion, and a subsequent drop in ATP production and energy crisis follows (94).

Mitochondrial dysfunction has been implied in the pathophysiology of traumatic brain injury (TBI) (9, 77, 95-98) and ischemic stroke (99), but also of neurodegenerative disorders such as Alzheimer's Disease, Parkinson's Disease, Huntington's Disease, and amyotrophic lateral sclerosis (11-14, 89, 100). However, it has only been sporadically described in ICH (5, 6). Mitochondrial dysfunction has increasingly become a target for potential drug development (77, 101-103) and may be a possible target for future neuroprotective drugs to treat ICH. As will be described in later sections, cerebral microdialysis (CMD) can be used to monitor the metabolic state of brain tissue and thereby mitochondrial function.

2.3 Current treatment guidelines

The treatment guidelines briefly outlined below are based on guidelines published by the American Heart Association in 2015 (43) and by the European Stroke Organisation in 2014 (44). The Swedish guidelines for stroke care are in accordance with the American and European guidelines (104).

2.3.1 Clinical presentation and diagnosis

ICH normally presents with symptoms including neurological deficits, headache, vomiting, seizures, reduced level of consciousness, and possibly neck stiffness caused by IVH. Immediate neuroimaging with non-contrast CT is needed to confirm the diagnosis, and to identify ICH volume, location, presence of IVH, and of any subarachnoid blood which could suggest an aneurysm (if cisternal) or CAA (if cortical) (105).

An underlying vascular lesion, such as an aneurysm, arteriovenous malformation or dural fistula, vasculitis, or posterior reversible encephalopathy syndrome, is found in 15% of adult ICH patients (106, 107). Risk factors for such an underlying vascular cause of ICH include age <65, peripheral or lobar ICH location, female sex, non-smoker, IVH, and no history of hypertension or coagulopathy (30, 43).

Spot-sign, visible on iodine contrast enhanced CT-angiography, is an indicator of active bleeding as it depicts ongoing contrast extravasation within the ICH (50). It indicates an increased risk of haematoma expansion and correlates to poor functional outcome (108). Further neuroimaging using MRI can be considered in the

subacute setting to determine underlying cause of ICH and presence and pattern of any cerebral small vessel disease (30).

After initial diagnosis the patient needs to be transferred promptly to the appropriate level of care. Treatment in a dedicated stroke unit correlates with lower mortality and less disability and dependency at 3 months compared to treatment in any other ward. This positive effect is particularly large for patients <65 years, unconscious patients and ICH patients (109). The positive effect on outcome of stroke unit care is likely due to structured protocols for monitoring and evaluating patients in order to prevent, detect and treat avoidable factors that otherwise lead to complications and secondary brain injury (109).

2.3.2 Medical treatment

In summary the medical management of the ICH patient aims to:

- limit haematoma expansion
- limit secondary brain injury by treating avoidable factors
- limit medical complications

2.3.2.1 Limit haematoma expansion

Haematoma expansion occurs in approximately one third of patients (49), and is associated with a poor prognosis (110-112). Factors consistently shown to contribute to haematoma expansion include raised SBP and coagulopathy (50, 113-116).

Large RCTs have been conducted to investigate the impact of intensive blood pressure lowering on haematoma expansion and functional outcome following ICH. The INTERACT2-trial of mild to moderate ICH, compared aggressive lowering of SBP (<140 mmHg) to standard care (<180mmHg) and showed that intensive SBP lowering was safe and lead to modest improvements in functional outcome (117). The international treatment guidelines were amended to include recommendations of lowering SBP to <140 mmHg within one hour of admission (43, 44). After the publication of these guidelines, however, another large RCT was published (ATACH-2), also comparing intensive SBP lowering (<140 mmHg) to standard care (<180mmHg). This study showed no difference in clinical outcome between groups but did, however, show an increased rate of adverse events in the intensive SBP lowering group (118). It has subsequently been suggested that SBP should not be lowered too aggressively; a target of 130-140 mmHg within 6 hours of ICH onset is recommended (30) in patients who do not have specific contraindications. Such an approach is safe, feasible, and may improve outcome. Only limited evidence exists,

however, for the effect of SBP lowering in sub-groups of ICH patients such as those presenting <1h after onset, those subjected to neurosurgery or those on anticoagulant medication (30).

Coagulopathy is associated with both haematoma expansion and poor outcome, and patients with a severe coagulopathy or thrombocytopenia should receive appropriate coagulation factor replacement therapy or platelets, respectively (43). The effect of vitamin-K antagonists (VKA) should be reversed using both 4-factor prothrombin complex concentrate (4-PCC) and vitamin-K if international normalised ratio (INR) is >1.4. Novel oral anticoagulants (NOAC) should be reversed using anti-dote if available (idarucizumab for dabigatran and possibly andexanet- α for factor Xa inhibitors), and otherwise receive 4-PCC. Patients on antiplatelet therapy at the time of ICH onset have worse outcome than patients without antiplatelet treatment (41). However, the PATCH study recently showed that platelet transfusion was associated with even worse outcome in ICH patients on antiplatelet therapy (66). In contrast, these patients benefit from treatment with tranexamic acid (TICH-1 study) contrary to the entire group of ICH patients (119). Therefore, tranexamic acid but not platelet transfusion should be considered for patients who suffer an ICH whilst on antiplatelet medication.

2.3.2.2 Limit secondary brain injury

Secondary brain injury is defined as the exacerbation of the primary brain injury sustained at the time of ICH onset, and can be caused by avoidable factors such as hypoxia, hyperthermia, convulsive or non-convulsive seizures, hyper- and hypoglycaemia, elevated ICP, and low CPP. Patients should be monitored to avoid, detect and treat such avoidable factors and this is best done in a dedicated stroke care unit (43, 44).

ICP should be monitored in poor grade patients, patients with clinical evidence of possible transtentorial herniation or increased ICP, and patients with IVH (43, 44). Furthermore, liberal use of continuous EEG monitoring is recommended and any clinically or electrographically detected seizures should be treated with antiepileptic drugs (43, 44). Blood glucose levels should be monitored and both hyper- and hypoglycaemia should be carefully avoided. Available guidelines do not state a specific target level, however, there is recent evidence that tight glycaemic control, advocated in critical care patients, may be detrimental to neurocritical care patients (120-123). This is probably due to a more frequent incidence of hypoglycaemic episodes and emphasises that hypoglycaemia should be avoided at all times.

2.3.2.3 Limit complications

Implementation of evidence-based interventions in order to limit complications has a positive effect on functional long-term outcome of ICH patients (124). Up to 50% of deaths in the first week following stroke can be attributed to medical complications. The most common medical complications following ICH are respiratory or infectious including pneumonia, aspiration, respiratory failure/distress, pulmonary edema and sepsis (43). Other common complications include deep vein thrombosis (DVT), myocardial ischemia, acute kidney injury, hyponatremia, gastrointestinal bleeding, impaired nutritional status, and post-stroke depression (43). Infections in airway, urinary tract, or surgical site should be actively sought and treated promptly. Patients should be treated with pneumatic intermittent compression to avoid DVT and, as soon as it is deemed safe, with low-molecular-weight heparin (125, 126). Dysphagia should be evaluated in conscious patients whereas in mechanically ventilated patients steps should be taken to minimise the risk of ventilator associated pneumonia. To detect medical complications and treat them as they arise is vital to improving patients' outcome following ICH.

2.3.2.4 Neuroprotective drugs

Despite promising results in animal models, no pharmacological therapy with neuroprotective action has translated to clinical benefit for ICH patients. This may partly be due to large heterogeneity among ICH patients compared to the well-controlled animal models. Nevertheless, there are some promising trials of new therapeutic agents targeted at reducing PHE or oxidative stress (64, 75). These include the iron chelator deferoxamine (DFX), now undergoing clinical trials (127, 128), which has been effective in both rat and pig-models, and shown to reduce PHE in ICH patients (129). Another promising agent is the PPAR- γ agonist *pioglitazone*, an antidiabetic drug, which can modulate phagocytosis, oxidative stress and inflammation after experimental ICH, and is currently being tested in the clinical setting (130). Numerous other compounds, such as the microglial inhibitor and iron-chelator *minocycline* and the sphingosine 1-phosphate receptors analogue *ingolimod*, have shown promising results in preclinical models or small pilot-studies (70).

2.3.3 Surgical treatment

Although surgery may be life-saving in some patients, the role of surgery for the majority of ICH patients remains a subject of debate. In the European guidelines for ICH treatment there is a weak recommendation that early surgery could be considered for patients GCS score of 9-12 (44). The American guidelines conclude that the

evidence does not support any recommendation on surgery for supratentorial ICH (43).

The reason for this lack of clearly defined role for surgery in the ICH treatment guidelines is a paucity of RCT based evidence. Although RCTs provide the highest level of evidence, such a study design presents certain challenges in the surgical setting (131). Nevertheless, RCTs comparing surgery to medical treatment in ICH patients have been undertaken over the years, including several small RCTs (132-137) and two highly influential large ones which have impacted on the role of surgery in the clinical management of ICH patients (138); the STICH (2005) (139) and STICH II (2013) (140) trials.

In surgical RCTs aiming to clarify the benefit of surgical procedures, patients are commonly included based on the concept of *clinical equipoise*. This means that patients otherwise eligible for inclusion are excluded if the clinician decides that one treatment option is either superior or futile to the other (141). The result is often the exclusion of young, otherwise healthy, patients or the very elderly from such studies (23) thereby limiting the generalisability of results. Evidence from study designs other than RCTs, such as non-randomised prospective studies or well-designed observational or even retrospective studies, should also merit consideration for decision making in ICH treatment.

Evidence for surgical treatment of ICH patients is summarised in the following sections. Since haemoglobin is distinctly neurotoxic (142) it follows intuitively that removal of a blood clot from the brain parenchyma should be beneficial for recovery of brain function and plausibly improve overall functional outcome. However, apart from as a potentially lifesaving treatment, the role and benefit of surgery for ICH remains uncertain.

2.3.3.1 Craniotomy

Craniotomy is a surgical procedure whereby access to the ICH is provided through a free bone flap, followed by a corticotomy, and tissue dissection to reach the blood clot which is subsequently removed by irrigation and suction (143). The STICH and STICH II studies, comparing early surgery to initial conservative treatment, were unable to show a benefit of surgery on functional outcome.

The first study, STICH, allocated >1000 patients to either early surgery or initial conservative treatment. Of patients allocated to early surgery 26% had favourable outcome at 6 months compared to 24% of initially conservatively treated patients ($p=0.414$).

Pre-specified subgroup analysis of surgically treated patients in STICH showed that patients with GCS 9-12, lobar ICH <1 cm from the cortical surface and no IVH had better outcome (139). Therefore, the STICH II trial (140) compared early surgery to initial conservative treatment in patients with GCS 9-12, 10-100 mL lobar ICH, and no IVH. *Ca* 600 patients were allocated to either early surgery or initial conservative treatment. Of patients randomised to early surgery 59% had an unfavourable outcome at 6 months, compared to 62% in patients randomised to initial conservative treatment ($p = 0.367$). In conclusion both these large clinical trials showed at best a modest result of surgery on functional outcome, none of them reaching statistically significant difference between groups. It has been suggested that the negative results may be explained by lack of power to detect small differences, or a large cross-over rate (*ca* 25%) from initial conservative treatment to surgery. It is equally plausible, however, that surgery for ICH is not effective in improving functional outcome.

Contrary to the negative results of the STICH trials two meta-analyses of RCTs comparing surgery to medical treatment have shown overall benefit of surgery, but with large data heterogeneity (144, 145) thus the uncertainty continues.

As the STICH trials included patients based on clinical equipoise inclusion was narrow, and the generalizability of the results is therefore limited. Whether or not surgery is helpful for the entire group of ICH patients is difficult to answer with a study design based on clinical equipoise. Furthermore, patient inclusion to STICH in Sweden was limited where two centres evaluated 38 patients eligible for inclusion but only recruited 8 patients.

As mentioned, the STICH trials particularly lack inclusion of young patients. Observational studies specifically of young ICH patients have shown a three times higher 3-month mortality in conservatively treated patients compared to surgically treated (146), and reduced mortality in surgically treated patients (147). Retrospective studies have their limitations, but they do provide “real-life” clinical results of surgically treated patients. One such retrospective single-centre study found a favourable outcome ($mRS \leq 3$) in a majority of ICH survivors and only 20 % mortality at long-term follow up (52). Another recently published single-centre retrospective analysis of ICH patients undergoing surgery, however, found a poor long-term outcome ($mRS 4-6$) in 76% of all ICH patients and in 86% of patients with supratentorial deep-seated ICH (54). Another study specifically of young (16-49 years) ICH patients showed 5- and 10-year survival of 93% and 88% respectively, and unfavourable functional outcome ($mRS 2-5$) in 49% of ICH patients surviving beyond one month (51).

2.3.3.2 External ventricular drain

An external ventricular drain (EVD) can be placed into the lateral ventricle of the brain for purpose of ICP monitoring, CSF drainage, hydrocephalus treatment or administration of intraventricular fibrinolysis in IVH (148-151). Large IVH components with a <30 mL parenchymal ICH can be treated by intraventricular fibrinolysis (using for example recombinant tissue plasminogen activator; rtPA), in accordance with the protocol for the phase III trial Clot Lysis: Evaluating Accelerated Resolution of Intraventricular Haemorrhage (CLEAR III) (152). The CLEAR III study showed a large effect on time to IVH clearance and to open ventricles, but no improvement on long-term functional outcome. Furthermore, confirming the findings of previous studies (153, 154) intraventricular fibrinolysis did not reduce the need for permanent ventricular shunting (155).

2.3.3.3 Minimally Invasive Surgery

Explanations for the modest effect of surgery for ICH may include that the surgical approach inflicts additional trauma to the already injured brain. To limit such surgical trauma minimally invasive surgery (MIS) techniques can be used. MIS includes procedures such as stereotactic puncture and drainage, with or without administration of local clot fibrinolysis, endoscopy assisted surgery, and other techniques aiming to minimise surgical access trauma (156). The phase II trial (MISTIE II) of stereotactical application of a catheter within the ICH followed by local administration of rtPA showed positive effects on PHE formation and ICH residual volume and found the technique to be safe (157), however, the recently published phase III trial (MISTIE III) showed no positive effect on functional outcome (35).

Various methods of MIS are commonly used in China, and several studies of variable design have been published lately. These include a recent review and meta-analysis of 5 RCTS and 9 prospective non-randomised studies comparing MIS to craniotomy that showed MIS to be superior in terms of mortality, rate of re-bleeding and rate of good recovery (158). A recent retrospective study comparing three surgical methods (endoscopic surgery, minimally invasive puncture and drainage, and craniotomy) in moderate basal ganglia ICH concluded that endoscopic surgery was safer than both craniotomy and minimally invasive puncture and drainage, and had greater improvement in neurological outcome with similar mortality rates (159). Furthermore, a small RCT of frameless stereotactically guided endoscopic ICH evacuation compared to conservative treatment (ICES) showed a higher percentage of favourable outcome in the surgically treated patients (42.9% versus 23.7%), however, not reaching statistical significance ($p = .19$) (160).

Challenges to MIS include having less control of the surgical area and any potential perioperative bleeding compared to during a craniotomy. In addition, when MIS is combined with local fibrinolysis, clot resolution takes time. In the MISTIE II trial the catheter was left in place for 72 hours, and only 10% of patients achieved >80% clot clearance within this time (157). The slow and partial removal of the blood clot allows for initiation and propagation of secondary injury processes triggered by the ICH. Therefore, a MIS technique which combines swift removal of the blood clot, which a craniotomy would provide, with a minimally traumatic access would be a preferred method. To date, this may be represented by endoscopic surgery or similar methods which provide a minimal working channel for ICH access (156) whilst still allowing prompt clot removal.

2.3.3.4 Decompressive hemicraniectomy

To date the evidence for decompressive hemicraniectomy (DC) in ICH is limited (161-163). A recent small RCT randomizing 30 patients to either craniotomy with ICH evacuation or DC found no significant difference in mortality or Glasgow outcome scale at 6 months (164). An RCT comparing DC to best medical treatment (SWITCH) is currently ongoing (NCT02258919).

2.3.3.5 Surgery in summary

In conclusion, although surgery can be life-saving for selected ICH patients, it does not result in improved functional outcome for the majority of ICH patients. MIS may in the future present the best option if it can allow access to the ICH via the route least traumatising to the already injured brain, combined with swift removal of the blood clot.

Apart from injury inflicted by the surgical trauma, there may be other explanations for the poor results of surgery for ICH. Secondary injury processes initiated by the ICH may persist after removal of the blood clot. Describing the pathophysiology of the secondary injury processes of ICH better may facilitate discovery of new targets for therapeutic intervention. It is likely that a combination of surgery and medical treatment is necessary to halt the secondary injury processes and bring about improvements in functional outcome for ICH patients.

2.4 Neurocritical care of ICH patients

In the neurocritical care (NCC) unit ICH patients are treated according to protocols that strive to detect and correct avoidable factors which could, if left untreated, lead to ongoing secondary brain injury. The multimodal monitoring commonly used includes measuring ICP, jugular venous saturation, arterial blood pressure and saturation, cerebral brain tissue oxygenation, cerebral metabolism monitoring by microdialysis, continuous electroencephalogram monitoring (cEEG), and pressure reactivity index (PRx) calculation to evaluate autoregulation (149-151, 165). Protocols aim to individualise and titrate treatment in order to maintain normal ICP, normotension, normovolaemia, normoventilation, normothermia, normoglycaemia, and adequate cerebral perfusion pressure (CPP). The NCC protocol employed in our department is outlined in chapter 4.1.3 on page 37.

2.4.1 Cerebral blood flow

Cerebral blood flow (CBF) is expressed as mL of blood that pass through 100g of brain tissue per minute, normally ≈ 50 mL/100g/min (166). If CBF drops below a critical threshold the neurons will malfunction. Initially the electrical impulse firing will stop and the cells will go into a hibernating state. Although there is no absolute threshold for this, it is considered to be approximately 18-23 mL/100g/min, with a different threshold in grey and white matter, respectively (167). Cells can survive in this state, for a shorter period of time, if CBF is subsequently restored. However, if CBF drops further still (<10 mL/100g/min) ion pumps will fail, cell membrane integrity will be lost and cell death ensues. From this state the cells cannot recover, even if CBF is restored.

CBF is regulated by various mechanisms including metabolic coupling, neural control, partial pressure of carbon dioxide in blood and pressure autoregulation (166, 168). CBF regulation following ICH has been the subject of debate. There is a zone of hypoperfusion immediately surrounding an ICH (7, 169-171), however, whether this hypoperfusion is evidence of ischemia or merely indicative of tissue hypometabolism has not been settled. Evidence from positron emission tomography (PET) and single photon emission computed tomography (SPECT) studies investigating the relationship between CBF and metabolism (4, 7, 63, 172) tends to favour the latter. More research is needed to characterise CBF following ICH and its relationship to cerebral metabolism.

2.4.2 Cerebral microdialysis

Cerebral microdialysis (CMD), which was developed in the 1970s (173), was first introduced in neurocritical care in the 1990s (174) and has now become an integral tool in the multimodal monitoring of the injured brain (175).

2.4.2.1 Basic principles

The basic principle of CMD involves insertion into living tissue of a catheter with a tip consisting of a tubular semi-permeable membrane. The catheter is perfused with fluid, at a constant rate; the perfusate. Molecules diffuse from the extracellular fluid, across the membrane, into the perfusate fluid, which is subsequently collected in microvials as dialysate fluid. These microvials are changed at regular intervals (Figure 2.6) and the dialysate can be analysed for low-molecular-weight metabolites bed-side in the NCC unit.

Figure 2.6 Basic principle of cerebral microdialysis (CMD).

Schematic description of CMD showing a) the catheter and catheter tip with a semipermeable membrane which allows diffusion of solutes across from the tissue to the dialysate (red arrows) as well as in the opposite direction (green arrows) depending on the concentration gradient. B) Dialysate fluid is collected in microvials which are changed regularly; every other hour in our department. Figure reproduced with permission from Zhou, T. & Kalanuria, A. *Curr Neurol Neurosci Rep* (2018) 18: 101.

The physical laws governing the transfer of analytes from the tissue to the dialysate are diffusion and convection. The aim is to keep convection to a minimum by eliminating both osmotic and hydrostatic gradients. Osmotic gradients are minimised by adding colloid or an osmotic agent to the perfusate, and hydrostatic gradi-

ents are minimised by attaching the collection vials to the patient's head, thereby avoiding any push-pull-effect. Furthermore, employing relatively low perfusion rates ($<2 \mu\text{L}/\text{min}$) minimises the effect of ultrafiltration driven by convection (176). When convection is minimised diffusion becomes the driving force of the transfer of analytes from the tissue to the dialysate fluid. Several factors govern the rate of diffusion of analytes across the membrane and thereby their concentration in the dialysate.

The concentration of an analyte in the dialysate ($C_{\text{dialysate}}$) is always a fraction of the true concentration in the sampled tissue (C_{tissue}), and the ratio between these is known as the relative recovery (RR)(177), expressed as

$$RR = \frac{C_{\text{dialysate}}}{C_{\text{tissue}}}$$

RR is dependent on several factors including those intrinsic to the analyte, the CMD membrane, the perfusate and the sampled tissue. Factors intrinsic to the analyte include its molecular weight, hydrophobicity, and three dimensional structure. All of these affect an analyte's diffusion coefficient and tendency to adsorb to the tissue matrix, CMD membrane or tubing (19). Factors intrinsic to the CMD membrane, including material and pore size, will also affect the analyte-membrane interaction and the analyte's RR. CMD-membranes have a determined pore size, known as the molecular weight cut-off (MWCO) of the membrane, also referred to as liquid permeability. The MWCO is not an absolute measure of the actual pore size, but rather a statistical measurement of the probes sampling efficiency, meaning that the probe membrane is impenetrable to 80-90 % of molecules of that MWCO or larger (19). Molecules larger than the specified MWCO of the membrane can also be present in the dialysate but in small amounts. The MWCO of CMD membranes most commonly used in clinical routine is either 20 kDa or 100kDa (16, 17, 178). Furthermore, the RR is directly proportional to the area of membrane available for diffusion; a larger area generates a higher RR. Perfusate composition also affects RR as diffusion is driven by concentration gradients. Lower perfusate flow rates generate higher RR at the cost of smaller sample volume per time unit. Finally, RR is also affected by factors intrinsic to the sampled tissue such as its tortuosity; rate of analyte clearance through uptake and metabolism; pH; and temperature (176, 179) (Figure 2.7). The tortuosity is a measure of the complexity of an analyte's route of diffusion, around for example impermeable cell membranes, on its way to the dialysate fluid (176, 179, 180) and can have a substantial effect on diffusion rate and RR.

Figure 2.7 Tissue factors influence relative recovery (RR).

RR is determined by $C_{\text{dialysate}}/C_{\text{tissue}}$. $C_{\text{dialysate}}$ is dependent on several factors intrinsic to the sampled tissue, such as tortuosity (red dashed line), as well as uptake and metabolism (black dashed line).

To summarise, the factors affecting an analyte's resistance to diffusion along a path from the undisturbed tissue to the dialysate fluid, meaning the total resistance to diffusion (R_{tot}), can be expressed as the sum of the resistance of three compartments: sampled tissue (R_t), membrane (R_m) and perfusate (R_p). This can be expressed as:

$$R_{\text{tot}} = R_t + R_m + R_p$$

In the *in vivo* situation R_t is typically greatest, whereas in the *in vitro* setup R_t is often negligible and instead the main part of the resistance is constituted by R_m . The bigger part of R_p is often the concentration gradient. In addition, once an analyte has made its way into the dialysate fluid it can adhere to tubing, collections vials or other surfaces, thereby decreasing the actual measured concentration of the analyte in the final sample (181).

Apart from the routinely monitored metabolites glucose, lactate, pyruvate, glycerol and glutamate, CMD can also be used to sample macromolecules such as chemokines, cytokines, and other peptides and proteins. These are potential biomarkers provided they can be measured accurately and repeatedly (182). Biomarkers can be investigated in tissue or in a variety of bodily fluids such as serum,

plasma, urine, saliva, tears, cerebrospinal fluid, or microdialysate (183, 184). The use of microdialysis has specific advantages including sampling directly in the extracellular matrix where cell-to-cell interaction and cross-talk takes place (185).

Biomarkers are increasingly used as clinical and diagnostic tools but also as surrogate endpoints in clinical research. The development of a new biomarker often begins with a screening process comparing levels of potential biomarkers in healthy controls compared to individuals with a particular disease or condition followed by a process of validation and evaluation (186). Proteomic techniques, covered in section 2.4.3.1, are useful in such a wide screening process. Biomarkers can also be used as markers of pathophysiological processes, as exemplified by levels of cytokine release in inflammation or amyloid- β or tau in neurodegeneration. Care must be taken not to equate correlation with causation, and biomarker results must be interpreted with caution.

2.4.2.2 Low-molecular-weight metabolites - Reference values

Metabolites routinely monitored to evaluate the energy metabolic state of brain tissue include glucose, lactate, and pyruvate. Glycerol and glutamate are considered markers of cell membrane degradation and excitotoxicity, respectively (187). Urea is often used in the clinical routine as an internal reference (188). Recovery of these low-molecular-weight metabolites is around 70% in a standard clinical setting when using a 10 mm 20 kDa MWCO membrane and 0.3 $\mu\text{L}/\text{min}$ flow rate of artificial CSF (189). Base line values of these metabolites are not possible to obtain from the brains of normal healthy subjects as CMD is an invasive method. There is, however, a study of 9 patients undergoing neurosurgical procedures of benign lesions in the posterior fossa, where a CMD catheter was placed in the right frontal cortex at the time of surgery. The patients were then monitored with microdialysis during surgery, anaesthesia and a wakeful post-operative period (190). The results of this study, which were recently validated (191), constitute the entire basis for what is referred to as normal baseline values in the literature (Table 2.2). Consensus on levels of low-molecular-weight metabolites considered clearly pathological, signalling metabolic distress in the sampled tissue, are glucose $<0.2 \text{ mmol/mL}$, and LPR >40 , whereas a glucose $<0.8 \text{ mmol/mL}$ and LPR >25 are considered warning signals (Table 2.2) (16).

Table 2.2 Normal and pathological levels of routinely measured low-molecular-weight metabolites.

Normal reference values, as well as warning signs and pathological levels, for low-molecular-weight metabolites as suggested by the Consensus statement of the 2014 International microdialysis forum.

Metabolite	Normal reference values, mean (SD) (190)	Warning sign (16)	Pathological (16)
Glucose (mmol/L)	1.7 ±0.9	<0.8 mmol/L	<0.2 mmol/L
Lactate (mmol/L)	2.9 ±0.9	-	>4.0 mmol/L
Pyruvate (μmol/L)	166 ±47	-	-
Lactate-pyruvate ratio (LPR)	23 ±4	>25	>40
Glycerol (μmol/L)	82 ±44	-	-
Glutamate (mmol/L)	16 ±16	-	-

As the LPR is a ratio, an increase can be due to either the numerator increasing or the denominator decreasing, which represents two distinctly different situations. These two situations have been defined as either a type 1 or a type 2 LPR elevation and correspond to a metabolic pattern of ischemia versus a pattern of mitochondrial dysfunction, respectively.

2.4.2.3 LPR elevation; ischemia or mitochondrial dysfunction

The two different situations of raised LPR represent two ends of a spectrum. Type 1 LPR elevation is characterised by low pyruvate and corresponds to a pattern of ischemia in the tissue, further characterised by a low glucose level. The type 2 LPR elevation is characterised by either normal or high levels of pyruvate in conjunction with increased lactate and reflects a pattern of mitochondrial dysfunction. Both situations may render the same numerical LPR but by different mechanisms. There are an increasing number of reports of a type 2 LPR elevation, indicative of mitochondrial dysfunction rather than ischemia, in various acute brain injury conditions (8, 10, 168, 192-196).

Brain tissue metabolism can be characterised by its redox state which is dependent on the cellular respiration. Cellular respiration is divided into three interconnected processes that convert glucose to energy; (1) the glycolysis, (2) tricarboxylic acid (TCA) cycle and (3) oxidative phosphorylation (Figure 2.8). Glycolysis takes place in the cytosol and results in 2 ATP molecules per glucose molecule. The TCA cycle generates another 2 ATP, but it is the oxidative phosphorylation which gener-

ates the vast majority of ATP. Both the TCA cycle and the oxidative phosphorylation take place within the mitochondria.

Figure 2.8 Overview of ATP production.

Under normal conditions glucose is metabolised to pyruvate by (1) glycolysis in the cytosol. Pyruvate then enters the mitochondrion where it is oxidised to Acetyl-CoA which is utilised in (2) the tricarboxylic acid (TCA) cycle and generates substrate for (3) the oxidative phosphorylation which is where the vast majority of adenosine triphosphate (ATP) is produced. When the mitochondria do not function (illustrated by dashed red lines), pyruvate accumulates in the cytosol, some of which is converted to lactate by lactate dehydrogenase (LDH). NAD/NADH = Nicotinamide adenine dinucleotide; FADH/H₂ = flavin adenine dinucleotide.

In the absence of oxygen, pyruvate will be anaerobically metabolised, within the cytosol, into lactate by lactate dehydrogenase resulting in 4 ATP per glucose compared to 36-38 ATP per glucose under aerobic conditions. Anaerobic metabolism thereby generates a high level of lactate and a low level of pyruvate in the extracellular fluid, leading to a high lactate/pyruvate ratio (LPR), the classic state of ischemia named a type 1 LPR elevation. In a situation where oxygen and glucose are available but the mitochondria are not functioning, intermediates of the steps prior

to the TCA cycle and the oxidative phosphorylation will accumulate. Since there is no shortage of substrate in this situation the glycolysis will run at an accelerated pace in an attempt to compensate. Pyruvate, the end product of glycolysis, accumulates to normal or above normal levels, and lactate can more than double (90). This will also result in an elevation of the LPR, but a so called *type 2 LPR elevation* where pyruvate remains normal or high. Measuring metabolites with CMD allows for the distinction between type 1 and 2 LPR elevations (8) and can thus give indications of whether the metabolic disturbance in the tissue is characterised predominantly by ischemia or mitochondrial dysfunction.

Metabolic crisis due to mitochondrial dysfunction has been indicated in animal studies of acute brain injury (197, 198) and in clinical studies of patients with TBI (192, 193, 195), SAH (10), malignant media infarction (194), or meningitis (196). However, studies evaluating mitochondrial function in ICH patients are scarce. One study where CMD-catheters were placed in the perihemorrhagic zone (PHZ) surrounding surgically evacuated ICHs showed a pattern of metabolic crisis suggestive of mitochondrial dysfunction rather than ischemia (6). In this study, however, neither the CBF nor cerebral tissue oxygenation was analysed. In addition, no comparison was made to metabolite levels in uninjured brain tissue. In support of mitochondrial dysfunction in ICH, a biopsy study of perihemorrhagic tissue from patients undergoing ICH evacuation showed evidence of impaired mitochondrial respiration (5).

In summary, although mitochondrial dysfunction seems an important secondary injury process in several acute brain injury conditions, its prevalence following ICH remains unknown.

2.4.3 Cerebral microdialysis for sampling macromolecules

With the introduction of large pore size catheters (100 kDa) CMD can now also be used for sampling macromolecules (199, 200). There are no previous studies of sampling protein biomarkers using CMD specifically in ICH patients, although ICH patients are part of the case mix in some NCC studies (18, 178, 201, 202). Several biomarkers have been evaluated in CMD-derived samples from TBI and SAH patients including: neurodegenerative markers (203) tau (204, 205), neurofilament light (NfL) and heavy (NfH) (206, 207), amyloid- β (205, 208, 209), glial fibrillary acidic protein (GFAP), S-100B, ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1), neuron-specific enolase (NSE) (203), cytokines, matrix metalloproteinases (MMPs) (210), and vascular endothelial growth factor (VEGF) (211, 212). These biomarkers have not yet been studied in ICH patients. By sampling biomarkers with CMD in

ICH patients we may improve our understanding of the acute tissue response to ICH and to related surgery.

2.4.3.1 Proteomics

Proteomics refers to the large-scale study of proteins. The term first appeared at the end of the 1990s as a counterpart to genomics which is the large-scale study of genes. The proteins expressed in a given cell, region, tissue or body fluid under determined conditions make up the proteome; a term analogous to the term genome. The study of proteomics can be applied to both physiologic and pathologic processes, to recognition and discovery of biomarkers, as well as to the study of effects of drugs or treatments (213). The aim of proteomics is to grasp and describe the proteome in an unbiased manner, without an *a priori* formed hypothesis (214).

The two most commonly used proteomic approaches are the gel-based and the liquid chromatography (LC) based approach. The gel-based approach involves separating proteins by two-dimensional gel electrophoresis (2-DE) prior to identification with, for example, mass spectrometry. The LC-based approach (also known as shotgun), has no protein separation step prior to digestion, but rather the proteins are digested directly in the sample and the peptide mix is identified using for example mass spectrometry to derive peptide sequences. This is then followed by database searching and protein identification by inference. The LC-based approach dominates the field of proteomics due to ease of implementation.

In summary, the mortality and morbidity of ICH remains high and there are limited treatment options. Surgery has not been shown to improve functional outcome, and clear clinical guidelines on surgery and post-operative treatment of ICH are lacking. Secondary injury mechanisms triggered by the blood are important in the ongoing brain injury following ICH, and these are not necessarily halted by the surgical removal of the blood clot. One possible secondary injury mechanism implicated following ICH is mitochondrial dysfunction leading to energy crisis in the tissue. Tissue metabolism can be monitored by cerebral microdialysis (CMD) which can indicate a metabolic crisis caused either by ischemia or mitochondrial dysfunction. CMD can also be used to sample proteins such as cytokines or other biomarkers.

3 AIMS

The general aims of this doctoral thesis were to characterise aspects of the pathophysiology of surgically treated ICH as well as to elucidate the clinical management of ICH across neurosurgical centres in Sweden.

Specific aims addressed in this thesis were:

- to characterise regional blood flow and the energy metabolic pattern in the vicinity of a surgically evacuated ICH (Paper I)
- to describe protein expression in the perihemorrhagic zone compared to seemingly normal cortex following surgical evacuation of ICH (Paper II)
- to describe differences in proteome profile following surgical evacuation of ICH in three compartments: microdialysate, microdialysis membrane and CSF (Paper III), and
- to describe similarities and differences in indications for surgery, treatment strategies and outcome among ICH patients undergoing surgical treatment at the six neurosurgical centres in Sweden (Paper IV).

4 METHODS

This section outlines patient inclusion and data collection followed by a description of neurocritical care protocol, surgical procedures and cerebral blood flow measurements. The microdialysis protocol is described, as are the methods for analysing low-molecular-weight metabolites. Employed definitions of lactate/pyruvate ratio elevation types are outlined. Gel-based and liquid-chromatography-based proteomic approaches are covered, and finally a description of the relevant statistical methods is included.

4.1 Patients

4.1.1 Inclusion and exclusion criteria (Papers I-IV)

Adult patients (>18 years), undergoing surgical evacuation of spontaneous supratentorial ICH at the Neurosurgical department of Linköping University Hospital, Sweden from November 2014 to November 2016 were conveniently recruited to Papers I-III and data was prospectively collected. Surgical and clinical decisions were made by the consultant neurosurgeon irrespective of inclusion in the study. Exclusion criteria were marked pre- or perioperative coagulopathy or inability to locate next of kin for consent. The study protocol for Papers I-III was approved by the regional ethics board in Linköping, Sweden (decision number 2014/236-31). Recruited patients could not themselves consent to inclusion, thus a written informed consent by proxy was obtained from the patient's closest relative. Five patients were included both in Paper I (total n=12) and II (total n=10), and three patients in Paper I, II and III. Thus, in total, 17 patients were included in Papers I-III.

Paper IV included 578 consecutive adult patients with supratentorial ICH undergoing surgical treatment at the six neurosurgical departments in Sweden (Lund, Gothenburg, Linköping, Stockholm, Uppsala and Umeå) during a five year period (2011-2015). Patients with a first-listed ICD-10 diagnosis code I61.0-9 were evaluated for inclusion. Patients with infratentorial ICH (cerebellum and brainstem), and ICH related to trauma, neoplasm, cerebral aneurysm, vascular malformation, venous sinus thrombosis or a haemorrhagic transformation of ischemic stroke were excluded, as were patients who did not undergo surgery. The study protocol for Pa-

per IV was approved by the regional ethics board in Uppsala (Dnr 2017-119), and each participating centre had local approval from the Head of Department.

4.1.2 Data collection (Papers I-IV)

Data was collected on parameters including: patients' age, gender, and past medical history; Glasgow coma scale motor response (GCS-M) or RLS-85; pupillary size, asymmetry and reaction to light; arm and leg paresis/paralysis; dysphasia or aphasia; and length of stay (LOS) in the neurocritical care (NCC) unit. Third cranial nerve compression suggestive of transtentorial herniation (defined as decreased level of consciousness in combination with uni- or bilateral pupil dilatation) was documented. Type of surgical procedure and number of reoperations was noted. The post-operative use and duration of ICP monitoring, CSF drainage, and mechanical ventilation was recorded. Outcome was determined according to mRS at 3-6 months post-surgery in Paper I-III and 30-day mortality in Paper IV.

All pre-operative CT scans were evaluated for ICH location, presence of hydrocephalus (215), IVH according to LeRoux severity scoring system (216), and ICH volume using the ABC/2 technique (217, 218). Lobar location of ICH was defined as an ICH with maximal diameter in frontal, parietal, temporal or occipital lobe or reaching <1cm from the cortical surface (52, 219), and deep-seated ICH as originating from the basal ganglia and thalamus (52, 139, 220). All patients in Paper I-III underwent a CT-angiography pre-operatively to rule out a structural cause of the ICH.

Parameters for data collection were chosen through a combination of existing evidence in the literature, and clinical experience. A pragmatic approach was adopted in the multi-centre nationwide review (Paper IV) on which parameters were feasible to collect from six different health care regions using disparate patient records systems. Where only RLS-85 scores were reported these were translated to GCS-M scores (see Table 4.1).

Table 4.1 Translation RLS-85 to GCS-M.

RLS-85	GCS-M
1	6
2	6
3	5
4	5
5	4
6	3
7	2
8	1

4.1.3 Neurocritical care protocol (Paper I-III)

Patients were managed post-operatively in the neurocritical care (NCC) unit in accordance with a standardised treatment protocol based on multimodal physiological and neurological monitoring to detect and act on signs of secondary insults.

Multimodal monitoring in our department entails monitoring arterial blood pressure, oxygen saturation, core body temperature, respiratory frequency, end tidal carbon dioxide, ICP, blood glucose, and cerebral metabolism with cerebral microdialysis. Frequent arterial blood gases are analysed to titrate the mechanical ventilation. Target paO_2 is >10 kPa and pCO_2 4.2-5.3 kPa. Blood glucose levels are monitored every 30-120 minutes and target level is normoglycaemia (6-8 mmol/L) with great care taken to avoid hypoglycaemia. ICP is monitored in unconscious or sedated patients and target ICP is <20 mmHg, using a tiered approach of measures to lower ICP as necessary.

Corticosteroids are not administered. Intermittent pneumatic compression is employed to avoid DVT, and LMWH is administered as soon as deemed safe, normally >48 hours post-surgery. In patients with worse than expected neurological status, where non-convulsive seizures can be suspected, continuous monitoring with electroencephalogram (cEEG) via scalp electrodes is employed, and the results are interpreted by a neurophysiologist. Documented seizure activity results in administration of antiepileptic drugs.

4.1.4 Surgical procedures (Paper I-III)

In our department surgical evacuation of an ICH via craniotomy is indicated in patients that are salvageable and display:

- depressed or deteriorating level of consciousness
- signs of ICP elevation, and

- where CT scan reveals an ICH (either lobar or in basal ganglia but not thalamic) causing mass effect.

Rescue therapy with hyperventilation and Mannitol to lower ICP can be used as bridging therapy before surgery. Additional vascular imaging with CT-angiography is performed when there is an increased risk of structural vascular cause of ICH (factors outlined in 2.3.1 on page 14).

In patients included in Paper I-III the craniotomy was performed by the neurosurgeon on call, in a standard neurosurgical operating theatre, through a free bone flap, followed by dural opening, corticotomy and subsequent microsurgical evacuation of the ICH by suction and irrigation. Haemostasis was achieved without cauterisation as far as possible. Care was taken not to further traumatise the perihaemorrhagic tissue. The corticotomy was performed in a non-eloquent area, kept as small as possible, distance from cortical surface to clot and trauma caused by surgical access was minimised. Hand-held retractors were preferred over fixed to avoid unnecessary tissue compression. Following evacuation of ICH any residual cavity was filled with saline, microdialysis catheters and ICP monitor were implanted, the bone flap was fixed back into place, the wound was closed and the surgery was concluded.

Depressed level of consciousness or significant mass effect prior to surgery was an indication for monitoring ICP post-operatively, either by an EVD or an intraparenchymal pressure monitor. The EVD was inserted under direct visual control during open surgical evacuation of the ICH, or according to anatomical landmarks contralateral to the ICH.

At the time of surgery, one CMD catheter was placed adjacent (<1 cm) to the ICH cavity (perihemorrhagic zone catheter; PHZ) and the other catheter was placed, either via the craniotomy or via a separate burr hole, in seemingly normal cortex (SNX) in a functionally silent, non-eloquent area of the brain (Figure 4.1).

Figure 4.1 Pre- and post-operative CT scan

A) Pre-operative CT scan of 51-year-old male patient with a left-sided deep-seated ICH. B) Post-operative CT-scan showing the cavity of the evacuated ICH (*), the tip of the PHZ-catheter (closed arrow), and the SNX-catheter (open arrow). Figure adopted with permission from Tobieson *et al.* (2018), *Neurosurgery*.

CMD catheters were inserted at a 45 degree angle through the cortical surface after a small punctate incision was made in the arachnoid and pia mater, and both the PHZ and SNX catheters were intended to cover the cortex-white-matter-junction of their respective areas. Bipolar cauterisation at the point of catheter insertion was avoided to minimise artefacts. Catheters were tunnelled through the skin and attached securely to the scalp. The collecting vials were attached to the patients head bandage to avoid hydrostatic push/pull-effects. The location of each CMD-catheter was confirmed on follow-up CT scans visualising the gold marker in the catheter tip, and the distance to the ICH cavity was determined. The ICH cavity was defined by post-operative changes such as a hypodense area, residual haematoma or fluid collection at the place for the ICH on the pre-operative CT scan.

4.1.5 Handling of samples (Paper I-III)

Blood and CSF samples were drawn every morning during the patient's treatment period in the NCC unit. The initial 2 mL of aspirated CSF were discarded in order not to sample CSF stagnant in the tubing. Serum, plasma and CSF samples were immediately sent to the hospital laboratory, centrifuged for 10 min at 1800 x g at

4°C, and the supernatant was subsequently decanted and stored in 1 mL aliquots at -86°C awaiting further analysis.

CMD samples were analysed for low-molecular-weight metabolites bed-side at the NCC unit. The remaining ca 30µL dialysate fluid was then immediately stored on ice, subsequently transferred first to -20°C freezer, and then stored in Eppendorf vials at -86°C awaiting further analysis.

CMD membranes were removed bed-side at the NCC unit by a neurosurgeon. The membrane and a small part of the tubing was cut into a polypropylene test tube, immediately placed in the -20°C freezer and subsequently transferred to storage in -86°C until further analysis.

4.2 Cerebral blood flow (Paper I)

For Paper I cerebral blood flow (CBF) was measured using Xenon enhanced computed tomography (Xe-CT; n=10) or, when Xe-CT could not be performed, computed tomography perfusion (CTP; n=2). CBF investigations were performed at day 0-2 post-surgery (CBF1), and repeated at day 3-6 post-surgery (CBF2).

4.2.1 Xenon-enhanced computed tomography

Xe-CT involves performing CT before and after the patient receives Xenon gas added to the inspired air. Xenon is a metabolically inert compound that acts as a contrast agent by freely diffusing into the tissue, thereby illustrating tissue perfusion. During the Xe-CT CBF investigations, patients were sedated and mechanically ventilated. Physiological parameters, use of muscle relaxant, sedatives and inotropic drugs administered during CBF measurements were recorded.

Figure 4.2 Xe-CT evaluation of CBF.

Xe-CT scans of 51-year-old patient showing (a) 20 automatically generated regions of interest (ROI), and (b) two manually outlined ROI centred on the CMD catheters. Figure adopted with permission from Tobieson et al. (2018), *Neurosurgery*.

Xe-CT exams were performed using a portable CT scanner (CereTom o-NL3000-001, Neurologica, Boston, USA) and a gas mixture of 28% Xenon was added to the ventilator air/O₂-mixture. A standard four-level Xe-CT CBF exam using 10 mm spacing was performed and a modified Kety-Schmidt method was used (221, 222) to calculate mean CBF (mL/100 g/min) (223, 224) which was then visualised using a colour coded image. Twenty evenly distributed regions of interest (ROI) were automatically generated in each of the four levels (Figure 4.2a), and an area surrounding each CMD-catheter (CMD-ROI) was manually outlined (Figure 4.2b). This CMD-ROI was $\approx 200 \text{ mm}^2$ to allow robust mathematical CBF calculations (224). Confidence intervals for CBF calculations were mathematically derived, visualised and inspected, and any ROI with artefacts was excluded from analysis.

The potential sources of error of CBF calculations based on Xe-CT include CT noise, low Xenon supply (<24%), compromised tissue enhancement effect, and motion artefacts. CT noise can contribute significantly to error in CBF evaluations (225). In addition, a potentially significant source of error is variation in Xenon tissue enhancement effect, which is dependent on the CT scanner setting. Optimal setting is 95-100 kVp resulting in tissue enhancement by around 5-6 HU (226), which is still a poor signal to noise ratio, since CT noise is around 3 HU. A kVp of 120 and a 27% Xenon supply, similar to what was used in our protocol, has been

shown to result in tissue enhancement of only 3 HU (225), making CBF calculations more sensitive to CT noise and other sources of error. Finally, motion artefacts result in a geographic distortion of the shape of the measured area although not of the calculated CBF value (225). Nevertheless, motion artefacts can result in the software discarding that scan, which could render the entire exam invalid; thus motion artefacts must be carefully avoided (226).

4.2.2 Perfusion Computed tomography

CTP was performed when Xe-CT could not be achieved ($n=2$), due to the patient being extubated, or physical limitation of the portable CT-scanner combined with patient body composition. CTP images were obtained according to a protocol used in clinical routine (227, 228). Images were reconstructed into 10 mm thick slices and further analysed using a deconvolution-based algorithm. Perfusion data was presented as colour-coded maps depicting CBF, CBV and MTT. Image quality was evaluated by a neuroradiologist and a ROI pattern corresponding to that of the Xe-CT examinations was then manually outlined.

Potential sources of error of CTP derived CBF include patient motion, choice of ROI for measuring arterial input function (AIF) and the effect of laterality of AIF. Current post-processing software can minimise the impact of these sources of error. Furthermore, CBF can be calculated either by a non-deconvolution or a deconvolution algorithm, of which the latter is less prone to error and was preferred (229). There are several studies validating and standardising the results of CTP based measurements of CBV and CBF (229) and showing adequate correlation between CBF calculations derived from CTP and Xe-CT (230, 231), thus the CBF data was pooled before statistical analysis.

4.3 Microdialysis (Paper I-III)

The 71 High Cut-off Brain MD Catheter with a 10 mm, 100 kDa nominal molecular weight cut-off polyarylethersulfone (PAES) membrane (M Dialysis AB, Solna, Sweden) (18, 178, 209) was used. Catheters were perfused with a water solution containing 5 % human albumin and the excipients sodium chloride, N-acetyl-DL-tryptophan and caprylic acid (Albunorm, 50g/l, Octapharma AB, Stockholm, Sweden), at a rate of 0.3 $\mu\text{L}/\text{min}$ using the 106 MD pump (M-Dialysis AB), as per standard protocol in our department. The first microvial was discarded after 60 minutes according to instructions from the manufacturer and in line with consensus praxis

(16). Microvials were changed and analysed every other hour, which is different to most standard protocols where vials are changed every hour (16, 165).

4.3.1 Interpretation of low-molecular-weight metabolites

Low-molecular-weight metabolites (glucose, lactate, pyruvate, glycerol, glutamate, and urea) were measured on ISCUSflex Microdialysis Analyzer® by enzymatic reagents and colorimetric measurements. The sample volume required for the different metabolites was 0.5 µL for glucose, 0.2 µL for lactate, 0.5 µL for pyruvate, 0.5 µL for glycerol, 1 µL for glutamate and 0.5 µL for urea leaving approximately 30 µL in the microvial after analysis.

The ISCUSflex Microdialysis Analyzer® performs an internal automatic calibration every six hours. The limit of detection for small molecular metabolites using ISCUSflex Microdialysis Analyzer® is 0.1 mmol/L for glucose and lactate, 10 µmol/L for pyruvate and 0.22 mg/mL for glycerol, and 1.0 µmol/L for glutamate.

In line with the latest consensus statement on microdialysis (16) the metabolite concentrations considered to represent normal, warning, or pathological levels are presented in Table 2.2.

Type 1 LPR elevation was defined, in line with previous literature, as LPR >25 in combination with pyruvate <70 µmol/L (10). There are several definitions of type 2 LPR elevations in the literature (10, 16, 194, 195, 197, 198, 232). We defined type 2 LPR elevation as LPR >25 or >40 combined with pyruvate within or above normal limit being either >120 or >70 which represents -1 or -2 SD of mean normal value, respectively (190). Type 2 LPR elevation is thus characterised by a high lactate and a pyruvate concentration within or above normal levels, to be contrasted with the ischemic situation where pyruvate is low. Glucose <0.8 mmol/L was considered 'warning level' and <0.2 mmol/L was considered 'pathological level' in accordance with consensus statement on microdialysis (16) (see Table 2.2).

4.4 Proteomics (Paper II & III)

Proteomics involves the analysis of the entire protein expression in a fluid or tissue, generally employing one of two approaches; gel-based or LC-based proteomics (Figure 4.3).

The gel-based strategy entails protein separation by 2-DE prior to identification, whereas the recently more commonly applied LC-based technique, also known as shot-gun proteomics, entails initial enzymatic digestion of proteins in a sample

followed by analysis of the resulting peptide mix using tandem mass spectrometry (MS/MS), and database search to identify the proteins originally present in the sample (233, 234) (see Figure 4.3). Protein composition in a microdialysate or CSF sample spans a vast order of magnitude, with the high abundant proteins being measured in the order of g/L, whereas low abundance proteins can be measured in pg/L or fg/L. In order to characterise all proteins in such a sample it is necessary to use a combination of techniques. In this thesis both a gel-based and LC-based approach was used.

Figure 4.3 Gel-based versus LC-based proteomics.

Schematic illustration of the two major proteomic approaches used to identify proteins in samples; in this example protein A and B. Gel-based approach entails initially separating proteins using 2-DE prior to protein digestion and application on MS/MS whereas LC-based approach (shotgun) entails digesting proteins directly in the sample without prior separation and subsequent application on MS/MS. Note that LC-based proteomics can only identify protein B as present in the sample mix, whereas 2-DE can identify both A and B. See also the discussion on protein inference problem on page 85. MS/MS = tandem mass spectrometry; MW = molecular weight; pI = isoelectric point; 2-DE = two-dimensional gel electrophoresis; LC = liquid chromatography. Figure reproduced with kind permission from Nesvizhskii *et al. Molecular & Cellular Proteomics*, 2005;4:1419-1440.

4.4.1 Sample preparation

4.4.1.1 Microdialysate (Paper II and III)

Two 2-hourly microvials were pooled for each analysis to obtain enough protein amount in each sample. Since 5 % albumin was used in the perfusate, resulting in a high amount of albumin in the samples, 40 μ L of each sample was applied to an Albumin & IgG Depletion column (GE Healthcare, Uppsala, Sweden), according to the manufacturer's recommendation. Samples were then desalted, lyophilised, and protein concentration was determined (235).

4.4.1.2 CSF (Paper III)

CSF samples were centrifuged and the supernatant decanted, in order to remove erythrocytes, prior to freezing and storage. Prior to analysis, CSF samples were applied to albumin depletion columns (Albumin & IgG Depletion SpinTrap, GE Healthcare), desalted with Amicon Ultra Centrifugal Filters, 0.5 mL Ultracel 3k (Merck Millipore Ltd, Cork, Ireland), and dried in SAVANT SPD 111V SpeedVac Concentrator. The dried samples were resolved in 150 μ L urea buffer solution and subsequently protein concentration was determined using 2D-quant Kit (GE Healthcare).

4.4.1.3 CMD Membranes (Paper III)

Membranes were cut into pieces and proteins were extracted by addition of urea buffer solution (8 M Urea, 4% (w/v) CHAPS, 65 mM DTT, 2% (v/v) pharmalyte 3-10, trace of bromophenol blue), incubation at room temperature and gentle shaking. An unused CMD membrane was used as a blank control. Protein concentration was determined by 2D-QuantKit (GE Healthcare).

4.4.2 2-DE-based proteomics

Two-dimensional gel electrophoresis (2-DE) proteome analysis entails the following steps (236):

1. sample preparation
2. 2-DE running
3. protein spot detection
4. image analysis of protein changes
5. excision of selected spots

6. destaining; reduction and alkylation; in-gel protein digestion and peptide extraction
7. peptide identification using either peptide mass finger printing (MALDI-TOF-MS) or peptide sequencing (MS/MS), and
8. protein identification by database search and bioinformatics.

Each step is described in detail in Paper II and III; however, some particulars will be noted here.

- Following preparation approximately 50 µg protein from each sample was analysed by 2-DE in accordance with the protocol described by Görg *et al* (237).
- Separated proteins were visualised by silver nitrate staining (238) after sensitisation with sodium thiosulphate and analysed as digitised images.
- The spot protein amount was measured as background corrected optical density, integrated over all pixels in the spot and expressed as integrated optical density (IOD).
- To correct for variability in staining, spot intensity was normalised against all detected spots in the gel generating ppm-values (parts per million) for statistical analysis. Furthermore, only spots which were present in more than 50% of samples of one group (PHZ-A; PHZ-B; SNX-A; or SNX-B) were included.
- Protein spots of interest were excised from the gel, destained using sodium thiosulphate and potassium ferricyanide, and digested in-gel with trypsin (239) and peptides were extracted.
- Peptides extracted from one spot were then applied to a nano-flow HPLC system in conjugation with a mass spectrometer with a nano-electrospray source.

Characteristic methodological strengths of 2-DE include (236):

- The physicochemical parameters of proteins are measured which can be used to confirm identification.
- It has a high resolving power whereby several thousand proteins can be resolved.
- Crude protein mixtures are tolerated thus further sample modifications can be avoided.
- High sample loads are possible.
- Isoforms and post-translational modifications are displayed.
- It is a non-destructive separation technique.

- It is an efficient fraction collector whereby proteins can be stored in the gel, protected from protease activity.
- Multiple detection is possible with staining or blotting.
- Sample multiplexing is possible with DIGE.
- With DIGE quantitative results can be obtained at a high confidence level.

In general, 10-15% of proteins will not be resolved by 2-DE due to their characteristics. Very basic proteins ($pI > 11$), hydrophobic proteins and high molecular weight proteins (> 200 kDa) will resist entry into a 2-D gel. Furthermore, low abundance proteins may be expressed below the level of detection, which for silver staining is 1-10 ng (240, 241).

Variability in 2-DE protein patterns may be caused specifically by:

- proximity to the acidic or basic end of the gel
- variable degree of protein loss; particularly of low-molecular-weight proteins
- solubility of samples
- co-migration of two or more proteins to the same spot
- temperature variations
- degradation products introducing new spots in old samples, and
- contamination of samples.

In general small amounts of variability in each step lead to a complex variation in spot sizes. Variation in the sample preparation procedure, amount of loaded protein, spot visualisation technique, spot measuring procedure, or batch-to-batch gel differences introduce variability in 2-DE patterns.

4.4.3 MALDI-TOF-MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) following in-gel digestion of a protein spot, allows measurement of peptide mass with high accuracy. This measured peptide mass is then compared to theoretical results derived from genomic databases, and peptides are matched to proteins. Sensitivity of peptide mass fingerprinting using MALDI-TOF-MS (Paper III) is in the femtomole to attomole range, and three peptides of high mass accuracy (ca 25 ppm) are most often sufficient to identify a protein (236). Peptide mass fingerprinting is easy and fast, but will not identify all protein spots, as posttranslational modifications (PTM), mutations, co-migration, or errors in databases may prevent a match. Non-identified spots can be subjected to MS/MS to obtain se-

quencing of the peptide which may allow identification. MS/MS is also needed for *de novo* sequencing of proteins or for proteins with high amount of PTM as peptide mass fingerprinting cannot be used for this. In Paper III a combination of MALDI-TOF-MS and MS/MS was used.

4.4.4 LC-based proteomics

LC-based proteomics entails the following steps (242) (Figure 4.4):

1. sample preparation
2. protein enzymatic digestion (for example with trypsin)
3. sample application on LC-MS/MS
4. matching of MS/MS spectra to individual peptide sequences from databases where best match is determined by scoring algorithms, and
5. using peptide sequences to infer which proteins were present in the sample.

Figure 4.4 LC-based proteomics approach.

Sample preparation, followed by protein digestion by for example trypsin, and sample application on MS/MS. Software is used to match MS/MS spectra to peptide sequences from database containing *in silico* digested proteins. Scoring algorithms allow best match to be determined thereby identifying individual peptide sequences. The set of peptide sequences are then used to infer which proteins may have been present. Unless a peptide is unique to only one protein there is always some uncertainty as to which protein a peptide originated from. LC = liquid chromatography; MS/MS = tandem mass spectrometry. Reproduced with kind permission from Cottrell (2011) Protein identification using MS/MS data. *Journal of Proteomics*, 74;10: 1842-1851.

Sensitivity of mass spectrometry is in the low femtomole levels (240, 243), however due to loss of peptides in each analytical step, in reality this level of detection is likely higher, probably in the range of nanomole. Sensitivity of MS/MS can be improved by using micro columns as less protein is then lost during column passage.

In this thesis MS/MS-spectra raw files were searched using Sequest HT in Proteome Discoverer (Thermo Fisher Scientific, San Jose, CS, USA; version 1.4.0.288) and peptide sequences against Swiss-Prot (Paper II-III) or NCBI database (Paper

III). Data was filtered at 1% false discovery rate (FDR). High peptide confidence was achieved by rank 1 peptides in the top scored proteins. SCAFFOLD (version 1.4.0.288; Proteome Software Inc., Portland, OR, USA) was used to filter identified proteins, based on minimum 1 unique peptide, 90% peptide identification probability by the Scaffold Local FDR algorithm, and 99% protein identification probability by the Protein Prophet algorithm, generating a <1% decoy FDR.

In the LC-based approach label-free quantitative analysis of peptides was performed by spectral counting analysis. To normalise run-to-run variations the normalised average total ion current was calculated for each protein and quantitative differences were statistically analysed using t-test. P values <.05 were considered statistically significant (although also p <.1 was reported in Paper II Table 4). Identified proteins were categorised according to gene ontology terms by using the STRING consortium database.

In Paper II the Swiss-Prot database was used which is a high quality, curated, non-redundant protein database within the UniProt Knowledgebase (UniProtKB). In Paper III the Swiss-Prot database was searched first and if this did not result in a protein match the redundant NCBI database was subsequently searched. A non-redundant database contains a consensus sequence for each protein and any known variants have been collapsed into one. This makes the database small, and searches fast, but proteins may be missed if they are low abundant and only represented by one peptide sequence. In contrast, in a redundant database, such as the NCBI database, all known protein sequences are explicitly represented. This makes the database larger, which means searches take longer and render a more difficult problem of protein inference (242) but the chances of a match are better.

As MS/MS-spectra identify peptide sequences and not proteins, the protein inference problem constitutes a challenge of MS/MS based protein identification, particularly in LC-based proteomics. Many peptide sequences are part of more than one protein. The guiding principle when inferring proteins from a sample is to apply the principle of a *minimal protein list*, or principle of parsimony or Ockham's razor. This means striving to compile a list of the minimal number of proteins that can account for the peptides observed in the sample (242). See further discussion on this in section 6.4.5 on page 85.

LC-based proteomics always involve some degree of uncertainty and ambiguity concerning protein identification (242). By using algorithms such as the Protein Prophet the peptide to protein match is converted into a probability score. This makes it easier to remove unreliable protein matches (242).

For large proteomic data sets, an estimation of FDR is recommended. This is achieved using a target-decoy search to validate results, meaning the search is repeated using the same search parameter settings but against a database where the sequences have been reversed or shuffled. The number of matches from this shuffled database gives an estimate of the number of false positives in the results from the target database. Decoy database searching is a means of result validation. Data in Paper II were searched with a decoy FDR of <1%. However, our highly stringent limits for protein identification mean that we are open to having made a type II statistical error in protein identification. In light of the ease at which large data sets are produced using proteomic techniques it would be a disservice to accept a lower threshold for protein identification. At the expense of having possibly missed a few low abundant but relevant proteins, we can safely say that the results of proteins identified to be present in our samples in Paper II and III are highly trustworthy.

4.5 Statistical methods

IBM SPSS Statistics 22 (IBM, Kista, Sweden), and SIMCA+ version 14 (Umetrics, Umeå, Sweden) were used for statistical analyses. Parametric data were presented as mean $\pm$ standard deviation, non-parametric data as median and range and/or inter-quartile range. The Shapiro-Wilk test was used as a numerical means of assessing normality. Two-tailed tests were used and a $p < .05$ was considered significant, however, results from univariate statistical analysis of quantitative LC-based proteomic data method were also presented for a p -value $< .1$ in Paper II. Data in microdialysis figures were presented, for clarity, using mean $\pm$ standard error of the mean (SEM).

4.5.1 Non-parametric tests

To calculate significant differences in gel-based proteomics a Mann-Whitney U-test was used. Wilcoxon's paired test was used for comparison of longitudinal samples. Correlations between microdialysis data, CBF measurements, and protein expression were investigated using Spearman's rank correlation. Kruskal-Wallis test of k -samples was used when comparing non-normally distributed continuous or ordinal variables between several independent groups.

4.5.2 Parametric tests

Paired t-test was used to compare means and Pearson's Correlation coefficient to investigate correlation in normally distributed data. Continuous outcome variables were compared among several groups using one-way-ANOVA, and the effect size

was investigated by Games-Howell post-hoc analysis. Proportions of categorical outcome variables were investigated using Pearson's Chi-square test and adjusted residuals designated effect location among groups.

4.5.3 Multilevel Mixed Linear model (Paper I-II)

A multilevel, hierarchical, mixed linear (MML) statistical test was used to investigate microdialysis data (Paper I, II) and CBF-measurements (Paper I) (244, 245). MML can be used to investigate whether auto-correlation is larger than cross-correlation in nested parameters (246). MML is preferred to a repeated measure ANOVA in the instances where the assumptions of a repeated measures ANOVA test are violated. This includes when time needs to be treated as a continuous variable, variables are nested, residuals are non-normally distributed, there is missing data, or there are differing number of repeats in a series.

Figure 4.5 Hierarchical data

Hierarchical data are suitable for analysis using a Mixed Model Linear (MML) approach. Individual data points are nested within higher levels. Ipsi = ipsilateral; contra = contralateral; ROI = region of interest.

In MML each data point is assigned to a higher hierarchical level within which all observations are nested. In our data patient-ID is such a higher subject level. The test then investigates if the variation in the data material is explained mostly by which patient-ID a data point belongs to or to the assigned fixed effect (which is the parameter being tested) such as for example ipsilateral versus contralateral hemisphere (Figure 4.5) (247).

Specifically, patient number was used as subject variable to indicate that random effects were correlated on this term. *Fixed effects* (hemisphere for CBF-measurements and catheter location (PHZ/SNX) for microdialysis data) were investigated for statistically significant effects, and patient number was used as a *random effect variable*.

4.5.4 OPLS-DA for ‘omics analysis (Paper II)

Multivariate statistical analysis (MVA) can be described as a useful group of tools for reducing the dimensionality of highly complex data sets and rendering the results more manageable both in terms of interpretation and visualisation (248). MVA builds a model describing the structured variation in a dataset, and it can handle all data (both numerical and categorical) in one ‘test’, thereby eliminating the problem of multiple comparisons. This is very useful when analysing large data sets such as ‘omics data where multiple testing would otherwise be problematic. Two common applications are principal component analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA).

PCA (illustrated in Figure 4.6a) is a so called unsupervised method, where there is no pre-specified definition of groupings in the material, but rather the data set is explored as a whole. The primary task of PCA in ‘omics research is mainly quality control including a search for outliers or overall trends in the data. This is then often followed by another step where the use of supervised methods becomes relevant.

Figure 4.6 Multivariate analysis workflow

(A) Multivariate models reduce the dimensionality of the data and describe the structured correlation in complex datasets by a set of latent variables. This can be compared to how the shadows of the 3-D cloud of data points can be projected onto a 2-D plane, exemplified by a red dot, projected onto the plane as a blue dot (dashed arrow). Doing this to each data point results in the PCA model. (B) The OPLS scores plot of a data set of healthy subjects (HC) and asthma patients (AC) illustrates the separation of subjects (each represented by a square or diamond). A clear separation is seen along the predictive component (x-axis) differentiating between groups. The y-axis displays the orthogonal component which explains the within-group variation. (C) The loadings plot shows the influence of different variables on the separation of the groups. Variables far apart on the x-axis strongly contribute to group separation (red) and may be potential biomarkers, whereas variables far apart on the y-axis contribute to within-group variation. (D) Variables with the highest potential as biomarkers can be identified by using variable selection parameters such as Variable Influence in Projection (VIP) and/or p(corr). PCA = principal component analysis; OPLS = orthogonal projection to latent structures. Reproduced with kind permission from Å. M. Wheelock and C. E. Wheelock, *Mol. BioSyst.*, 2013, 9, 2589.

Supervised methods (such as OPLS-DA, Figure 4.6b) focus the model on finding variables that are important for separation of predetermined groups in the data. This could be, for example, proteins separating healthy subjects from asthma patients or, as in Paper II, group PHZ-B from SNX-B. The proteins included in the final model are those that contribute most to group separation. These can be selected in several ways (248, 249) one of which is by retaining only variables with varia-

ble of importance in projection (VIP) >1 , as was done in Paper II. The CV-ANOVA with p -value $<.05$ was then used to cross validate the model (250). Model parameters, i.e. R^2 , Q^2 and CV-ANOVA p -value were reported.

5 RESULTS

This section outlines the results of Papers I-IV. Descriptive statistics of patients included in the prospective studies (Paper I-III) are summarised. This is followed by an account of the regional cerebral blood flow, and the metabolic state of the perihaemorrhagic tissue. The results of proteomics analysis of cerebral microdialysis (CMD) derived samples are presented, and related to findings of substantial protein adsorption to CMD membranes. Finally the differences and similarities in surgical treatment and neurocritical care of ICH patients in Sweden are summarised.

5.1 Patients (Paper I-III)

During the study inclusion time period (November 2014 to November 2016) 24 patients underwent craniotomy for spontaneous ICH at our department. Following screening, 17 patients (71%) could be recruited and included in Paper I-III: 12 patients in Paper I; 10 patients in Paper II; and three patients in Paper III. Five of the patients in Paper I were also included in Paper II, and all three patients included in Paper III were also included in Papers I and II.

Median age, in the 17 patients included in Paper I-III, was 62 (range 26-71) years. Mean ICH volume was 75.5 ($\pm$ 35) mL. Five patients had a lobar and 12 patients had a deep-seated ICH. Mean distance from the CMD-catheter to the ICH cavity was 6 ($\pm$ 4) mm for the PHZ catheter and 27 ($\pm$ 12) mm for the SNX catheter. Median GCS-M was 5 (range 3-6) on arrival, and 6 (range 3-6) on departure. The median length of stay was 5 (range 3-9) days. Median time from ICH onset to surgery was 10 (IQR 6-22; range 3-37) hours. The median time from ICH onset to start of CMD sampling was 16 (IQR 12-34; range 5-54) hours, and mean total time of CMD-sampling for all 17 included patients was 99.4 ($\pm$ 45) hours. The mean time microdialysis vials were stored in -20°C freezer was 180 ($\pm$ 102; range 9-359) days.

Patients were treated at the NCC unit according to a standardised protocol (see section 4.1.3). None of the patients underwent reoperation, and there were no instances of deep vein thrombosis, surgical site infection, post-operative meningitis or pneumonia documented during their NCC stay. Post-operative ICP-lowering treatment with osmotherapy was not required in any of the included patients, and there were no instances of documented epileptic seizures.

5.2 Cerebral blood flow following ICH surgery (Paper I)

The aim was to evaluate the perihemorrhagic regional cerebral blood flow (rCBF) following surgery for ICH. Eighteen patients were initially recruited to this study, however six patients were subsequently excluded due to either catheter malfunction ($n=3$) or that CBF evaluations were not performed according to protocol ($n=3$). CBF 1 was performed at $21 (\pm 10)$ hours and CBF 2 at $58 (\pm 16)$ hours using Xe-CT ($n=10$) or CTP ($n=2$). Median age was 64 (range 26-71) years. Ten patients were male and two were female. Median GCS-M score on arrival was 5 (range 3-6). ICH location was deep-seated in 10 patients and lobar in two patients. Median time from ICH onset to start of surgery was 14 (range 3-37) hours. One patient had a small post-operative epidural haematoma without mass effect which was treated conservatively.

rCBF was lower in the ipsilateral than the contralateral hemisphere (35.0 ± 21 vs. 38.0 ± 20 mL/100g/min, respectively, $p < .05$; Figure 5.1) at CBF 1, whereas, at CBF2 there was no difference between hemispheres (40.4 ± 20 and 39.5 ± 18 mL/100g/min respectively; $p = .25$; Figure 5.1). ROIs centred on the CMD catheters (see Figure 4.2 on page 41), revealed a significantly lower CBF in ROI_{PHZ} (25.7 ± 14 mL/100g/min) compared to in ROI_{SNX} (40.9 ± 20 mL/100g/min; $p < .05$) at CBF1, but no difference at CBF2 (36.5 ± 27 mL/100g/min in ROI_{PHZ} and 42.7 ± 30 mL/100g/min in ROI_{SNX}; $p = .426$).

Figure 5.1 CBF heat-map.

Colour coded heat-map of mean cerebral blood flow (CBF) for each region of interest (ROI). There was a significant difference between ipsi- and contralateral hemispheres at CBF1 but not at CBF2. * indicates $p < .05$. ipsi = ipsilateral hemisphere; contra = contralateral hemisphere; n.s. = not significant. Legend indicates mL/100g/min. Adopted with permission from Tobieson *et al.* (2018), *Neurosurgery*.

In summary, the rCBF adjacent to the ICH was lower than that of areas remote from the ICH at the first but not at the second CBF examination. The results show that rCBF in the vicinity of the evacuated ICH recovers following surgery and that although mean rCBF was below normal reference values it was well above the threshold for ischemia.

5.3 Microdialysis (Paper I & II)

The aim was to determine the metabolic pattern in the perihemorrhagic zone (PHZ) compared to seemingly normal cortex (SNX) following surgery for ICH. In total in Paper I and II, 7417 analyses in 1680 CMD samples were performed, including glucose (n=1503), lactate (n=1505), pyruvate (n=1544), glycerol (n=1507) and glutamate (n=1358). CMD data was initially edited and quality checked by research nurses in our NCC unit. Subsequently, five vials in Paper I and two vials in Paper II were excluded from analysis due to deviating urea values.

Figure 5.2 CMD low-molecular-weight metabolites.

Low-molecular-weight metabolites show (a) significantly lower glucose, (b) higher lactate, (c) lower pyruvate, (d) higher LPR, and (e) higher glutamate for PHZ compared to SNX, whereas there was no difference in (f) glycerol. Error bars represent standard error of the mean. * indicates $p < 0.05$. # indicates that the 95% confidence intervals of the area under curve do not overlap.

In Paper I mean concentrations of all CMD-derived low-molecular-weight metabolites apart from glycerol differed significantly between PHZ and SNX during the first 4-84 hours ($p < 0.05$; Figure 5.2a-f). Glucose < 0.8 mmol/L was more prevalent in

PHZ (48%) than in SNX (26%) and glucose was consistently lower in PHZ compared to SNX although both were above the warning level on average (Figure 5.2a). Glucose levels were pathological (<0.2 mmol/L) (16) in 9% of samples in the PHZ, compared to none of the SNX samples. LPR was elevated in the PHZ compared to the SNX ($p<.05$; Figure 5.1d). LPR was >25 in 70% of samples in the PHZ compared to 23% in the SNX ($p<.05$), and >40 in 38% of samples in the PHZ and 3% in the SNX ($p<.05$) despite the relatively low prevalence of critically low glucose values in both PHZ and SNX. The prevalence of a type 2 elevation was higher in the PHZ compared to the SNX, both when using LPR >25 and >40 as cut-off values and this difference persisted over time ($p<.05$; Table 5.1; Figure 5.3).

Table 5.1 Proportion of type 1 versus type 2 LPR elevation in PHZ and SNX at early and late time period.

		4-48 hours		49-84 hours	
		PHZ, n (%)	SNX, n (%)	PHZ, n (%)	SNX, n (%)
LPR > 25	Type 1 [†] LPR	57 (30)	42 (58)	23 (21)	18 (62)
	Type 2* LPR	133 (70)	30 (42)	85 (79)	11 (38)
LPR >40	Type 1 [†] LPR	42 (41)	3 (60)	19 (32)	8 (89)
	Type 2* LPR	61 (59)	2 (40)	41 (68)	1 (11)

Abbreviations: PHZ = perihemorrhagic zone; SNX = seemingly normal cortex; LPR = lactate/pyruvate ratio; [†] Type 1 LPR elevation defined as LPR elevation and pyruvate <70 $\mu\text{mol/L}$. * Type 2 LPR elevation defined as LPR elevation and pyruvate >70 $\mu\text{mol/L}$.

Figure 5.3 Proportion of type 1 versus type 2 LPR elevations.

The proportion of type 2 LPR elevation was persistently higher in the PHZ (indicated by red circles) compared to the SNX both in (a) samples with LPR >25 and (b) samples with LPR >40. LPR = lactate/pyruvate ratio; pyr = pyruvate; PHZ = perihaemorrhagic zone; SNX = seemingly normal cortex; 4-48 = hour 4-48 of study period; 49-84 = hour 49-84 of study period. * indicates $p < 0.05$. Figure adopted with permission from Tobieson *et al.* (2018), *Neurosurgery*.

In summary, analysis results of low-molecular-weight metabolites demonstrated a persistent metabolic crisis in the PHZ which was not present in the uninjured cortex. This metabolic crisis in the PHZ was mainly characterised by a type 2 LPR elevation, suggestive of mitochondrial failure, which is further supported by the findings of rCBF above ischemic thresholds.

5.4 Proteomics (Paper II & III)

The aim of Paper II was to determine the proteomic pattern of the PHZ, how it differs from SNX, and how the proteome evolves over time in these two locations.

The aim of Paper III was to investigate how the proteome profile differs between microdialysate, CMD membrane and CSF.

5.4.1 Proteome of microdialysate (Paper II)

Ten patients (9 male and one female) were included in Paper II. Median age was 57.5 (range 26-70) years and median GCS-M score on arrival was 5 (range 4-6). Eight patients had a deep-seated and two had a lobar ICH. Haematomas were evacuated at mean 13.5 (± 9.7) hours after ICH onset.

Using 2-DE, we identified and quantified 232 ± 31 different protein spots (Figure 5.4). Due to the use of 5% albumin in the perfusate, and despite our efforts to remove it, the results were heavily dominated by albumin. Thus the investigation was focused on proteins with a molecular weight < 50 kDa. Two proteins differed in integrated optical density (IOD) between the MD catheters at time A, and 12 proteins at time B ($p < .05$). Thirteen proteins were significantly altered between time A and time B in the SNX and seven in the PHZ, respectively ($p < .05$).

Figure 5.4 Typical 2-DE gel of perihaemorrhagic microdialysate.

A typical 2-DE gel result is shown with pI on the x-axis and molecular weight (Da) on the y-axis. White circles indicate spots with significant differences in IOD comparing PHZ to SNX ($p < .05$). Reproduced with permission from Tobieson *et al* (2019). *Scientific Reports* Vol 9, Article number: 3181.

Using nLC-MS/MS ca 800 proteins were identified, 76 of which were present in $\geq 50\%$ of samples in one of the four groups (PHZ-A, PHZ-B, SNX-A and SNX-B) and were further analysed statistically. Univariate analysis revealed one upregulated and two downregulated proteins in the PHZ compared to SNX at time A, whereas seven proteins were upregulated, and four downregulated in the PHZ compared to the SNX at time B. Quantity of the identified proteins was investigated also using OPLS-DA whereby 12 proteins were identified as most important for group separation between PHZ-B and SNX-B (Figure 5.5a-b).

Figure 5.5 OPLS-DA score plots.

(a) OPLS-DA model of the discriminant separation between PHZ (blue circles) and SNX (red circles) at time B (time interval 68-76 (range 50-62 to 70-80) hours post-ICH). On the X-axis the interclass discrimination is shown, and the Y-axis shows the intra-class discrimination of PHZ and SNX. (b) Loading plot corresponding to proteins with a variable of importance for projection (VIP) value >1. The circles correspond to the most important protein for PHZ and the squares indicate proteins important for SNX. OPLS-DA = orthogonal projection to latent structures discriminant analysis; PHZ= periahemorrhagic zone; SNX= seemingly normal cortex; VIP = variable of importance in projection. Reproduced with permission from Tobieson *et al* (2019). *Scientific Reports* vol 9, Article number: 3181.

Pathway analysis of all differentially expressed proteins showed that proteins involved with inflammatory response, regulation of endopeptidase and hydrolase activity were those linked to most significant pathways.

In summary, combining proteomic analysis with CMD derived sampling of proteins revealed distinctly different proteome profiles of the perihemorrhagic zone compared to uninjured cortex and these differences were particularly evident at the later time point.

5.4.2 Proteome of membranes (Paper III)

Three male patients, 49, 56 and 68 years old, were included, contributing in total five membranes (one was accidentally discarded at the NCC unit), six dialysate samples and three CSF-samples. Protein separation by 2-DE revealed a unique protein pattern for each of the three compartments; dialysate, membrane, and CSF (Figure 5.6).

Figure 5.6 Differences in proteome profile.

2-DE protein patterns of microdialysis catheter membrane after insertion in (A) seemingly normal cortex and (B) perihemorrhagic zone, (C) the dialysate samples from seemingly normal cortex, and from (D) perihemorrhagic zone, and (E) from cerebral spinal fluid. The circled areas indicate visual protein pattern differences between 2-DE gels. Tobieson *et al*, submitted manuscript.

Total protein concentration ($\mu\text{g}/\mu\text{L}$) was lowest in the membrane samples and highest in the dialysate samples. In contrast, CMD membranes had the highest number of spots (mean 448.2 ± 56) followed by CSF (mean 352.3 ± 73) and dialysate samples (mean 301.0 ± 28). Using LC-MS/MS we were able to identify 25 of 68 excised spots from the CMD membrane gel and 30 of 62 spots from the CSF gel.

High abundant plasma proteins such as haemoglobin, apolipoproteins and haptoglobin dominated results. However, there were also less abundant proteins present on the membrane which were not found in CSF or in microdialysate including: calmodulin-like-4 isoform CRA_c, BRICK1, mitochondrial import inner membrane translocase subunit Tim8 A, transthyretin, S100-A9, and Aldo-keto-reductase family 1 member C2. Distinct quantitative differences in integrated optical density were seen for numerous spots between the three compartments.

In summary, results show significant protein adsorption to the CMD membranes following implantation. Distinct quantitative differences were seen between the profile of membrane adsorbed proteins compared to the profile of microdialysate and of CSF. This emphasises the importance of analysing protein adsorption to CMD membranes.

5.5 Neurocritical care and surgery for ICH in Sweden (Paper IV)

The aim of Paper IV was to explore differences and similarities in indications for surgery, as well as operative, and post-operative care strategies in ICH patients among the six neurosurgical centres in Sweden.

Paper IV included 578 consecutive patients from six neurosurgical centres (353 (61%) males and 225 (39%) females), with a median age of 59 (range 20-85) years undergoing surgical treatment during 2011-2015. This constitutes approximately 5% of patients with supratentorial ICH in Sweden.

Table 5.2 Incidence of surgery for ICH per 100,000 person-years

	Lund	Gothenburg	Linköping	Stockholm	Uppsala	Umeå	<i>p</i>
All	1.26	0.75‡	1.53*	1.03	1.22	1.72*	<.001
Craniotomy	1.03*	0.47‡	1.07*	0.65‡	0.79	1.31*	<.001
EVD	0.22	0.28	0.47	0.38	0.43	0.41	=.086
* indicates significantly higher, ‡ indicates significantly lower than other centres. Abbreviations: EVD = external ventricular drainage							

The overall incidence of surgery for ICH and incidence of craniotomy with ICH evacuation differed among centres (Table 5.2). Patient age showed a bimodal distribution with one peak at 56 years and the second peak at 68 years (Figure 5.7).

Figure 5.7 Frequency distribution of age.

The histogram shows age distribution of the entire cohort of Paper IV. The frequency distribution resembles a bimodal distribution with two separate mean peaks at 56 and 68 years, respectively. N= number of patients.

Median ICH volume was 72 (IQR 54-98, range 15-240) mL for patients treated by craniotomy, 10 (IQR 4-26, range 0-225) mL for patients treated by EVD alone, and 60 (IQR 30-88, range 0-240) mL for the entire cohort of patients. A deep-seated ICH was seen in 341 (59%) patients, whereas 237 (41%) patients had a lobar ICH. Median GCS-M on arrival was 5 (range 1-6; IQR 5-6). Level of consciousness on admission was GCS-M1 in 8 (1%) of patients, GCS-M2 in 25 (4%), GCS-M3 in 28 (5%), GCS-M4 in 68 (12%), GCS-M5 in 217 (38%), GCS-M6 in 199 (34%), and missing in 33 (6%) of patients, respectively. Median time to surgery was 9 (IQR 5-20; range 0-312) hours. The median length of stay in NCC unit was 6 (IQR 3-11; range 0-34) days. A summary of ordinal and continuous variables is presented in Table 5.3 including a report on missing values.

Table 5.3 Descriptive statistics incl. missing values.

Variable	Median	25 th perc	75 th perc.	Obs. (n)	Missing (n)	<i>p</i> -value*
All patients (n=578)						
Age (years)	59	51	68	577	1	.148
ICH volume (mL)	60	30	88	544	34	<.001
PreGCS-M	5	5	6	545	33	.405
Time to surgery (hours)	9	5	20	509	69	<.001
LOS (days)	6	3	11	365	213	<.001
Patients treated by craniotomy and ICH evacuation (n=402)						
ICP monitoring (n=243 (60%), days)	6	3	11	243	0	.004
CSF drainage (n=109 (27%), days)	8	4	12	105	4	.037
Mechanical ventilation (n=316 (79%), days)	5	1	10	316	0	<.001
ICH volume (mL)	72	54	98	389	13	<.001
Patients treated by EVD alone (n=176)						
ICP monitoring (n=155 (88%), days)	10	4	16	155	0	<.001
CSF drainage (n=145 (82%), days)	11	5	17	143	2	<.001
Mechanical ventilation (n=143 (81%), days)	5	2	12	141	2	.005
Abbreviations: perc. = percentile; obs. = observations; PreGCS-M = pre-operative Glasgow Coma Scale Motor score; ICP = intracranial pressure; CSF = cerebrospinal fluid; EVD = external ventricular drain; LOS = length of stay. * <i>p</i> -value from independent samples Kruskal-Wallis test comparing distribution across all six neurosurgical centres.						

There was no significant difference among the centres in patients' age or pre-operative level of consciousness, although the proportion of patients displaying pupil abnormalities indicative of possible transtentorial herniation was higher in Umeå ($p < .05$). Furthermore, the profile of patients' co-morbidities was similar among centres, apart from a history of previous stroke which was more common in Stockholm than other centres. Differences were seen among centres in ICH characteristics including volume and proportion of deep-seated ICH ($p < .05$; Figure 5.8). Gothenburg operated on ICH volumes on average 23-35 mL smaller than the other centres. A higher proportion of deep-seated ICH was treated by craniotomy in Lin-

köping and lower in Stockholm compared to the other centres. Uppsala took on average 12 hours longer from ICH onset to start of surgery than Lund and Gothenburg did ($p<.05$).

Figure 5.8 Volume and proportion of deep-seated ICH.

(a) Haematoma volume in patients undergoing craniotomy and ICH evacuation was significantly smaller in Gothenburg than at other centres. (b) The proportion of patients undergoing craniotomy and ICH evacuation for a deep-seated ICH was higher in Linköping and lower in Stockholm than other centres ($p<.001$). * indicates adjusted residual $>\pm 2SD$. Reproduced with permission from Fahlström, A., Tobieson, L., Redebrandt, H.N. *et al.* (2019) *Acta Neurochir.*

Post-operative treatment, including the use and duration of ICP-monitoring, CSF-drainage, and mechanical ventilation, varied among centres ($p<.05$; Table 5.4).

Table 5.4 Neurocritical post-operative care strategies.

Proportion of patients in which the treatment option was used, N (%)							
<i>Patients treated by craniotomy and ICH evacuation</i>							
	Lund N=90	Gothenburg N=43	Linköping N=55	Stockholm N=74	Uppsala N=80	Umeå N=58	<i>p</i> - value
ICP	26(28)‡	12(28)‡	45(82)*	40(54)	67(84)*	53(91)*	<.001
CSF	15(16)‡	2(5)‡	12(22)	29(39)*	31(39)*	20(35)	<.001
Vent	60(66)‡	31(72)	48(87)	56(77)	67(84)	54(93)*	<.001
<i>Patients treated by EVD alone</i>							
	Lund N=20	Gothenburg N=26	Linköping N=24	Stockholm N=44	Uppsala N=44	Umeå N=18	<i>p</i> - value
ICP	17(85)	11(42)‡	23(96)	43(98)*	43(98)*	18(100)	<.001
CSF	16(80)	24(92)	20(83)	43(98)*	24(55)‡	18(100)*	<.001
Vent	12(60)	22(85)	19(79)	36(82)	37(84)	17(94)	.131
* indicates significantly higher and ‡ significantly lower than other centres. Adjusted residuals $\geq \pm 2SD$ are indicated by bold lettering. Abbreviations: N = number of patients; ICH = intracerebral haemorrhage; ICP = monitoring intracranial pressure; CSF = drainage of cerebrospinal fluid; EVD = external ventricular drain; Vent = use of mechanical ventilation.							

The 30-day mortality ranged between 10-28% with higher mortality in Linköping and Umeå and lower mortality in Lund compared to other centres ($p = .026$) but the effect size was small.

In summary, the nation-wide retrospective study of neurosurgical treatment of ICH patients in Sweden revealed a difference in the incidence of surgical intervention, but patients selected for surgery displayed similar clinical features. Differences among the centres were found in ICH-specific characteristics including haematoma volume and proportion of deep-seated ICH, and in the post-operative neurocritical care strategies.

Results in summary:

- Regional cerebral blood flow in the vicinity of an evacuated ICH normalised following surgery.
- A persistent metabolic crisis indicative of mitochondrial dysfunction was prevalent in the perihaemorrhagic zone.
- Proteins related to inflammatory response, regulation of endopeptidase and hydrolase activity were differentially expressed in the perihaemorrhagic zone.
- Protein adsorption to cerebral microdialysis (CMD) membranes was substantial.
- Patients' age, co-morbidity profile and pre-operative level of consciousness was similar among surgically treated ICH patients over a five-year period in Sweden.
- Differences were found in overall incidence of surgery for ICH, in the volume and location of ICH in operated patients, and in the post-operative neurocritical care strategies.

6 DISCUSSION

In this section, the pattern of metabolic crisis in the perihemorrhagic zone, indicative of mitochondrial dysfunction, seen despite a non-ischemic cerebral blood flow will be discussed. Then the results of the proteomic analyses, and their technical limitations, are critically evaluated. Implications of the variability in neurosurgical and neurocritical care among neurosurgical centres in Sweden are addressed. This is followed by a method discussion appraising the limitations and suggesting improvements of the methods used in this thesis.

6.1 Metabolic crisis in perihemorrhagic zone (Paper I)

Despite a normalisation of the rCBF in the PHZ following ICH surgery, a raised LPR indicating a persistent metabolic crisis was observed. This LPR elevation was predominantly of a type 2 suggestive of mitochondrial dysfunction.

Similar to our present results, previous studies on surgically and conservatively treated ICH patients have shown hypoperfusion linked to hypometabolism rather than ischemia in the area surrounding an ICH (7, 171, 251-256). Such hypometabolism could be explained by mitochondrial dysfunction (4, 6, 7, 172). A pattern of mitochondrial dysfunction has been previously observed in acute brain injury including in diffuse TBI (195, 257, 258), perilesionally in focal TBI (192), in SAH (10, 222), and in ischemic stroke (99, 194, 259, 260), but has not been thoroughly investigated in ICH patients.

One previous study used microdialysis placed in the vicinity of ICH following evacuation to investigate the metabolic state of the tissue (6), and found glucose within normal levels, which was interpreted as a sign of adequate CBF. The mean lactate was elevated, both in the first 12 hours and throughout the 3-day CMD-sampling period. In contrast, the LPR gradually normalised from an initial elevation. Although the pyruvate levels were not reported, the results imply that pyruvate increased over the study period, in order for the LPR to normalise. The authors interpreted these results as a metabolic derangement indicative of a biochemical penumbra, and speculated that mitochondrial damage could be involved since lactate remained high.

In further support of the role of mitochondrial dysfunction in ICH, impaired mitochondrial respiratory function was found in a study comparing perihemorrhagic tissue samples from six ICH patients to control tissue from patients undergoing elective neurosurgery for other conditions. The authors reported a trend towards more pronounced impairment in mitochondrial respiration with increasing time from ICH onset to surgery (5).

In Paper I we report on the LPR, lactate and pyruvate levels, and the prevalence of type 1 and 2 LPR elevations in accordance with the various definitions available in the literature (8, 10, 16, 168, 194, 195, 197, 232, 261). Observations in PHZ were compared to those seen in seemingly normal, uninjured cortex. However, without a biopsy of perihemorrhagic tissue we cannot with certainty state that the observed metabolic crisis was due to mitochondrial failure. Likewise, brain tissue oxygenation or PET measurements of oxygen extraction fraction could have further verified that the metabolic crisis observed in the PHZ was not caused by ischemia. Arguably, the finding of a CBF above ischemic thresholds, normalising over time post-ICH, suggests that ischemia in the PHZ was unlikely.

Although above ischemic thresholds, CBF was lower than normal, plausibly due to an effect of sedation used in the majority of patients (11/12) at time of CBF evaluation. The use of two different methods for determining CBF can be criticised, although there are studies showing reliable translation of CBF between Xe-CT and CTP (230, 231, 262). Failure to utilise CTP would exclude all extubated patients from the study protocol, since Xe-CT requires sedation and mechanical ventilation. Similarly, respiratory unstable patients, not able to undergo Xe-CT, would also have had to be excluded if CTP was not included in the protocol. Therefore, it was justified to utilise two methods to measure CBF. In addition, the use of MML statistical model diminishes any possible impact of using two methods.

The definition of type 1 versus 2 LPR elevation varies in the literature (8, 10, 16, 168, 194, 195, 197, 232, 261), which complicates interpretation and comparison of results among studies. Also, the classification of cerebral metabolism into only one of two possible patterns is an oversimplification. Cerebral metabolism is more likely described as a continuum between two extremes, represented by the two types of LPR elevation. Thus the metabolic state of the tissue can be characterised by the *degree* of ischemia versus the *degree* of mitochondrial dysfunction present at any given time. The metabolic state of the tissue can also potentially shift from one pattern to the other over time (168, 195, 261), and knowing which pattern predominates may be instrumental in targeting interventions to each individual patient.

As both CMD-catheters were typically placed through the cortical surface rather than through the surgical cavity, the distance from the cortical surface to the tip of the catheter should be similar in all catheters. Nevertheless, there were small differences among catheters in distance to cortical surface and angle of the CMD membrane observed on control CT, which could have led to the catheters monitoring areas with slightly differing proportion of white matter. This could affect both the low-molecular-weight metabolite results but also the diffusion rates of macromolecules as the tortuosity of the tissue differs between white and grey matter, a physical fact utilised in the diffusion tensor imaging (DTI) technique (263).

As no control subjects of conservatively treated ICH patients were included in Paper I, it is not possible to determine whether or not the persistent metabolic crisis in the PHZ is a lingering effect of the ICH despite surgery, or if the surgery itself causes or contributes to it. Furthermore, as CMD is a highly focal monitoring technique, we cannot verify that our results are representative for the entire PHZ.

Mitochondrial dysfunction following ICH could plausibly be triggered by an initial ischemia in the PHZ from compression by the blood clot on local vasculature (264), or by toxic effects of blood break-down products. Surgical removal of the ICH does not seem to be sufficient to amend the metabolic crisis indicative of mitochondrial dysfunction, but may need to be combined with pharmacological therapies to allow optimal tissue recovery.

As enzymes in the mitochondrial respiratory chain have different characteristics of light absorption depending on redox-state, near infra-red spectroscopy (NIRS) could be used to monitor mitochondrial dysfunction (168), and could even improve mitochondrial respiratory function through stimulation of cytochrome oxidase (265). Other plausible treatments for mitochondrial dysfunction include administration of succinate (266), cyclosporine A (102), methylene blue (265, 267, 268), a variety of antioxidants (103, 269) and stem cell transplantation of mesenchymal cells which donate healthy mitochondria to neurons (270).

6.2 Proteomic analysis of microdialysate and membranes (Paper II & III)

The results of Paper II show that there is a distinctly different pattern of protein expression in the tissue surrounding the evacuated ICH (PHZ) compared to seemingly normal, uninjured cortex (SNX). These differences include an upregulation of proteins involved in the inflammatory response, and in regulation of endopeptidase and hydrolase activity.

The acute and subacute inflammatory response was the most significantly up-regulated protein pathway in our analysis. This is supported by previous studies of ICH pathophysiology which have shown inflammatory processes to be active both in the acute and chronic phase (84, 271, 272) following ICH.

Our study is the first to characterise and compare protein expression in microdialysate close to and remote from an evacuated ICH. Previous proteomic studies of ICH include a study of plasma protein expression of eight ICH patients (273), which found 31 differentially expressed proteins compared to controls. Intriguingly, only one of these proteins was found in Paper II of this thesis. The lack of overlap of the results of that study (273) and our work indicate that the proteome profiles of blood and CMD are distinctly different following ICH.

A rodent study using proteomic analysis of brain tissue following experimental ICH found upregulation of the ERK1 and ERK2 signalling pathways. Upregulation of these pathways was not found in Paper II. Furthermore, none of the specifically identified proteins in rodents matched any of the proteins significantly altered in our material. This suggests a vast difference either in protein expression between rodents and humans following ICH, or in analytical methods between these studies.

There are several previous proteomics studies of plasma and CSF in various acute brain injury conditions such as TBI (274) and in spinal cord injury (275), although only a few in acute stroke research. Applying proteomics to CMD samples has been done sporadically, such as in TBI (276) and SAH (277). One study of the protein expression in cerebral microdialysate (CMD) of six patients suffering from acute ischemic stroke found distinct proteome patterns in the infarct core, the penumbra and the contralateral side (278). In another microdialysis study of three acute ischemic stroke patients, 27 proteins were identified and compared to proteins previously described in CSF. Ten mainly intracellular proteins were found only in microdialysate, although no conclusions were drawn as to their biological functions (279). Several of the 27 proteins were also seen in the results of Paper II and III of this thesis, illustrating that there are plausible similarities in the proteome profile of extracellular fluid in ischemic and haemorrhagic stroke patients.

Differences in protein concentration sampled by CMD may be caused by several mechanisms, apart from up- or downregulation of protein expression. These mechanisms may include edema formation affecting diffusion rates (72), breakdown of and subsequent leakage of blood-derived proteins into the interstitium (72, 79), impairment of the glymphatic system which affects protein clearance rates (280, 281), and diffusion of proteins from any ICH remnant into the interstitium (76, 282) (Figure 6.1). It is plausible that all of these factors contribute to the ob-

served results, and must be considered. Nevertheless, differences in protein expression may generate hypotheses for future investigations exploring proteins that could either prevent or ameliorate destructive processes or enhance endogenous neuro-protective ones.

Figure 6.1 Protein increase mechanisms

Several factors may contribute to increased protein concentration in the dialysate fluid apart from (1) increased expression by the viable cells in the tissue. These factors include (2) leakage of proteins from a compromised BBB, or protein diffusion into the tissue (3) from an ICH remnant, or (4) from necrotic cells and debris. It is likely that all these mechanisms contribute, to some degree, to our present results.

The time-period chosen for the second sampling was 72 hours post-ICH onset which corresponds to a clinically relevant time point when the PHE has developed and patients often deteriorate. Furthermore, the choice of 72 hours post-ICH onset allowed sufficient time to pass from the primary to the second sampling interval so that changes in protein expression had time to develop.

Due to the restriction in pore size of the CMD-membrane it is possible that we have failed to detect proteins that are larger than 100 kDa and of essential importance in the pathophysiology of ICH. Protein adsorption to CMD-membrane also means that some proteins evaded detection in the dialysate. Such proteins should, however, be detected through a separate analysis of membrane adsorbed proteins as shown in Paper III.

Tissue damage due to microdialysis catheter insertion can be either due to insertion trauma or effects of perfusion. Pre-clinical studies have shown that the catheter insertion trauma causes an acute but transient ischemia, disruption of endothelial cells, activation of astrocytes and microglia and a loss of both neurons and axons along the probe tracks (202, 283-288). Transient opening of the BBB occurs within 15 min of probe implantation but is no longer seen after 1.5 h (289). Perfusion then potentially further disrupts the tissue around the probe causing edema and necrotic neuron degeneration leading to decreased neuron density, axonal degeneration, swollen mitochondria, and deranged synapses (290) (see Figure 6.2).

Figure 6.2 Electron micrographs of perfused tissue

(a) Normal striatal tissue from rat for reference. (b-c) Following perfusion striatal tissue 100 μm from the probe edge shows dark degenerating profiles (*), disintegration of structural integrity, fewer synapses, and swollen mitochondria and dendrite profiles. Scale bars=1 μm . Symbols: open arrow = synapses; m = mitochondrion; g = glia; d = dendrite; t = terminal; s = spine. Adopted with permission from Clapp-Lilly *et al.* (1999) *Journal of Neuroscience Methods*, 90;2:129-142.

A proteomic study of changes induced in rat brain following insertion and perfusion of microdialysis probes showed that perfusion of artificial CSF increased ATP production compared to non-perfused tissue where it was persistently lower than in control tissue (291). Thus, it seems perfusion can improve function and contribute to tissue recovery following probe insertion trauma.

In this thesis a paired catheter approach was used, thus any artefacts of CMD catheter insertion trauma and perfusion would presumably be similar in both PHZ and SNX.

The use of albumin in the perfusate presented a challenge to both proteomic approaches, as the presence of high abundant proteins such as albumin hampers detection of low abundant proteins. Despite our efforts to remove it, albumin was still present in the samples and this interfered with the results. It is necessary to use a colloid in the perfusate when using high-molecular-weight cut-off membranes (100kDa), as the problem of ultrafiltration and fluid loss to the tissue otherwise becomes significant. Various Dextran molecules have been used as perfusate colloid (20, 178, 181, 292) some of which have been shown to leak across the membrane into the tissue (178), where they can generate an undesirable inflammatory response (20). Recently, perfusion fluid consisting of artificial CSF with 3% added Dextran MW 500 has been certified for clinical use (M-Dialysis, Solna, Sweden) and our department has now switched to using this perfusate.

Protein adsorption to CMD membranes has been shown previously (19, 20, 176, 284, 293, 294) although not following implantation in patients. The proteomic analysis of CMD membranes in Paper III showed, for the first time, that several proteins adsorbed to the membrane following sampling of human brain tissue and were not seen, or seen only in low amounts, in the dialysate.

In an *in vitro* study of proteins adsorbed to CMD membranes following dialysis of ventricular CSF pooled from 10 patients, 50% of proteins identified on the membranes were not identified in the CSF (293). Several methodological issues may contribute to these results, including the use of tryptic digestion of non-denatured membrane-bound proteins. This may have resulted in a partial elution of peptides from the membrane adsorbed proteins. Furthermore the use of a 3kDa spin filter after enzymatic digestion rather than before could result in the loss of several peptides and subsequent failure of protein identification. Nevertheless, this study (293) found protein adsorption to CMD membranes to be significant, similarly to Paper III of this thesis.

Proteomic analysis of microdialysate can generate hypotheses on novel therapeutic targets which may also have been implicated in previous studies of neuroprotective factors. Examples of upregulated proteins in Paper II that indicate neuroprotective function is transthyretin in the PHZ and dermcidin in the SNX. Previous preclinical studies have shown transthyretin to be neuroprotective following ICH (295-298) and dermcidin to be protective against oxidative damage in *in vitro* and in animal models (299-302). The role of such proteins in the pathophysiology of ICH needs to be further explored in future research.

It is plausible that protein adsorption could be decreased by surface modification of microdialysis membranes and tubing, as has been done in preclinical studies

showing improved recovery of for example cytokines (292, 303). Until such modifications become available for clinical use, however, the results of Paper III emphasise the importance of analysing protein adsorption to the CMD-membrane for a more accurate characterisation of the proteome profile.

6.3 Neurocritical care and surgery for ICH in Sweden (Paper IV)

Paper IV explored differences and similarities of surgical and neurocritical care of ICH patients among the six neurosurgical centres in Sweden during a five year period. Although there was a slight difference in the overall frequency of surgical intervention for ICH, similarities were seen among centres in patients' age, pre-operative GCS motor score, and co-morbidity profile. This suggests that indications and patient selection was similar nation-wide, was based on prognostic clinical features common to all types of acute brain injury, and not specific to ICH patients. Noticeably differences among centres were seen in ICH volume, proportion of deep-seated ICH, and post-operative management including use and duration of ICP monitoring, CSF drainage and mechanical ventilation. The observed variability illustrates that the lack of clear guidelines lead to variations in patient management.

Possibly, this variability contributed to the small differences in 30-day mortality observed between centres. The overall 30-day mortality in the entire cohort (16.7%) was lower than reported in previously published studies on surgically treated ICH (139, 140, 304, 305). Patients included in Paper IV were already considered to potentially benefit from surgery, and constitute about 5% of supratentorial ICH patients in Sweden. They do not represent the entire ICH population and any comparison would be precarious. Nevertheless, 30-day mortality in the cohort in Paper IV was significantly lower than predicted by the ICH score (306), which has been shown to be a reliable prognostic scoring scale for the entire ICH population (307).

No regression analysis of variables related to mortality was performed in this study. Thus, the reasons for the differences in 30-day mortality are unclear but may include a higher proportion of deep-seated ICH, or presence of clinical signs of transtentorial herniation which were more prevalent in the two centres with higher 30-day mortality. These two centres also had a slightly higher incidence of surgical intervention, suggesting they apply more liberal indications for surgery for ICH than the other centres do.

Apart from ICP monitoring, which is an integral part of the multimodal monitoring of comatose neurocritical care patients (149, 308) and recommended in ICH treatment (43, 44), existing guidelines are vague on surgery and post-operative care

strategies for ICH patients. Variability in ICH care, as shown in Paper IV, has also been previously shown in large international data (309) with surgery rates ranging between 2-74 % among countries. Such variability shows that a lack of clear guidelines translates into differing practices and variation in care provided to ICH patients.

Only a very small proportion (n=9, 1.6%) of all patients in the nation-wide cohort underwent surgery by a technique other than craniotomy for ICH evacuation, and these cases were largely endoscopy-assisted ICH evacuation, classed as minimally invasive surgery (MIS). There are no large RCTs evaluating outcome following endoscopy-assisted ICH evacuation compared to craniotomy. The small ICES study (160) compared endoscopic ICH evacuation to medical treatment but failed to show a statistically significant difference between groups. Recently published results of the MISTIE III trial, evaluating administration of alteplase locally in the ICH clot in >250 patients, did not show any benefit on functional outcome compared to best medical treatment (35). The standard mode of surgery for ICH, craniotomy, could change in the future if clinical trials of MIS for ICH show encouraging results. A recently published systematic review and meta-analysis of 14 RCTs comparing MIS to medical treatment or craniotomy showed a significant benefit of MIS on death or severe disability at long-term follow up. The beneficial effect was seen both for endoscopic surgery and stereotactic thrombolysis, and the chance of being functionally independent at follow-up was 2.2x higher in MIS group compared to other treatment. In addition, the chance of being independent at follow-up was 2.3x higher in MIS group compared to craniotomy (310).

Advances and improvement in surgical techniques often occur in an incremental manner which makes them difficult to investigate with study designs such as RCTs, commonly used to obtain a high level of clinical evidence. Nevertheless, as clinical experience and research data on MIS accumulates ICH patients may in the near future undergo MIS, to swiftly remove the ICH, combined with administration of a neuroprotective drug. Such a combination could significantly impact on the morbidity and mortality of this devastating disease.

6.4 Limitations

6.4.1 Patient inclusion

Paper I-III is based on a relatively small number (in total n=17) of non-consecutively recruited ICH patients. Consecutive patient recruitment would have been preferred

to guard against an uneven pattern of inclusion introducing confounding factors. In addition, only patients already subjected to surgery were included, thus further limiting the generalisability of the results.

The number of ICH patients undergoing surgery is low, which reduces the number of patients available for inclusion. Furthermore, the study design was highly complex, and invasive techniques were used, hence data gathered from these patients was difficult to generate, explaining why some patients were included in more than one paper.

6.4.2 Data collection

Information on ICH remnant following surgery and parameters to judge mass effect prior to and after surgery (basal cistern effacement, midline shift, presence of cortical surface sulci) was not collected. Equally, more data on medical management of patients in Paper IV would have been informative. For example, details on the blood pressure on admission and during the initial treatment phase would have added valuable information. In addition, the administration of intraventricular fibrinolysis was not noted and, although presumably infrequent, its use could be considered an important treatment parameter with possible impact on outcome.

The multi-centre study (Paper IV) presented particular challenges as all centres have different systems for keeping patient records, as well as different department structure and organisation. For example, the centres use different scales for documenting level of consciousness, thus full GCS scores were not available for all patients, in which case RLS-85 scores were translated to GCS-M. Furthermore, level of consciousness upon discharge was not comparable among the six centres since some reported discharge from NCC unit, whereas others from the general ward. Therefore we chose to exclude this parameter from our analysis.

Reliability of results in the nation-wide study (Paper IV) could have been increased had patient records and neuroimaging data from all centres been collected by one researcher, and subsequently validated by a second researcher. Here, a pragmatic study design was used, and data gathering was performed by up to three researchers at each centre, possibly leading to a degree of interrater variability.

In addition, the choice of outcome parameters can be criticised. The 30-day mortality is precise and reliable, although functional outcome may be an even more important factor to patients, families and treating clinicians. In Paper IV, a pragmatic approach was needed since not all centres had information on functional outcome, and we settled for 30-day mortality as the outcome measure.

The modified Rankin Scale (mRS), which was determined in Paper I-III at 3-6 months after ICH onset, is the most commonly used outcome scale in stroke research (55). However, it has been criticised on several points (57, 58), including low interrater reliability (57). Interrater reliability can be improved by employing a validated structured questionnaire and interview (311) as was done in Paper I-III. Another limitation of using mRS is the lack of patient reported outcome measures (PROM) such as measures of QoL (57). The questionnaire sent to patients in Paper I-III included EQ-5D and EQ-VAS, although this information was not analysed in this thesis. Yet another critique of the mRS is the lack of emphasis on neuropsychological deficits. Adequate long-term follow up of functional outcome following ICH should include measures of activities of daily living (ADL) function, along with PROMs and determination of any neuropsychiatric deficits. Preferably, this could be accomplished by a combination of a questionnaire sent to the patient and a visit to an outpatient clinic. In order to allow sufficient time for long-term improvement such an evaluation should be done at least 12 months after ICH onset (58). Future studies of long-term outcome following surgery for ICH are planned.

6.4.3 Microdialysis

The difference between the PHZ and the SNX catheters' distance to the ICH cavity was small, only a few millimetres, in some patients. Plausibly, between catheters placed at almost the same distance to an evacuated ICH large difference in metabolites or protein expression is not to be expected. Pathological processes induced by an ICH are likely occurring in relation to the distance from the ICH cavity. Analysing distance from CMD catheter to ICH cavity as a continuous variable rather than as a dichotomous classification (PHZ/SNX) based on intended placement was considered; however, due to the low number of patients any such correlation would be statistically uncertain.

The tissue in which catheters were placed could have been better characterised by performing a subacute MRI, but this was not feasible for logistic reasons. In addition, tissue outcome with an MRI at a later time point would have made such characterisation more reliable.

Microdialysis vials were changed every other hour, different to most protocols where vials are changed every hour. This means our low-molecular-weight metabolite results have poorer time resolution than hourly protocols, but we consider this to be an acceptable trade-off for lowering the work load of bed-side NCC staff. Changing vials every hour may, however, be preferable in terms of sample preservation, as this means each vial is kept at room temperature for a shorter time.

Storage time for microdialysis vials in the -20°C freezer was variable, and although we have no indication that this impacted on our sample quality, it is possible that a variable degree of degradation and evaporation would introduce variations in our results. However, since we used a paired catheter approach, the samples of which were transferred at the same time in each step, the effect of any such degradation or evaporation would be similar between the paired catheters.

6.4.4 2-DE Proteomics

Limitations to the 2-DE method include that it is time consuming, has limited dynamic range, does not work particularly well with hydrophobic proteins, and is essentially non-quantitative although quantitative read-outs can be achieved using digitised images. Furthermore, 2-DE has been criticised for low reproducibility, although the introduction of immobilised pH gradient (IPG) strips has greatly improved this. IPG strips containing fixed buffering groups allow high spatial resolution and high protein loading resulting in a better possibility of resolving low abundance proteins (236). Nevertheless, 2-DE technique tends to resolve a limited number of highly abundant proteins, involved in a limited number of cell functions and these are often described repeatedly; sometimes called the ‘deja-vue of proteomics’. Critiques view 2-DE proteomics as a screening process with high resolution and reproducibility, which is semi-quantitative, gives a label-free intermediate read out, but has limited analysis depth in terms of protein scope (312).

We chose to use silver staining in our 2-DE protocol. Silver staining is highly sensitive, and affordable, however, it has a short dynamic linear range ($0.04 \text{ ng/mm}^2 - 2 \text{ ng/mm}^2$) (313), thus care must be taken to load proper amounts of protein on each gel. There are various silver staining protocols (241, 313, 314) and we employed the protocol reported by Schevshenko, because this is more compatible with mass spectrometry as the glutaraldehyde step is omitted (243). However, silver staining is not specific to proteins, but stains also glycosylation and lipids thereby increasing spot intensity without higher amount of protein (241). Furthermore, silver staining is not easily compatible with mass spectrometry as the destaining protocol introduces compounds which can cause peptide modification such as the potassium ferricyanide (313). This makes it challenging to identify the protein in some spots, such as in Paper III where only approximately 40% of the excised spots were identified. Failing to identify a protein in what seems like a relatively strong spot can be explained by a combination of problems. Firstly non-specific staining of lipids and glycosylations may increase spot intensity of a low abundant protein. This protein can then be further depleted by loss of peptide in each analytical step, and

finally detection can be hindered by interference of the destaining protocol. An alternative would be to use fluorescent dyes, such as Sypro Ruby, which has a larger dynamic range, stains proteins specifically but is slightly less sensitive than silver staining, and less affordable.

6.4.5 LC-based proteomics

As most of the peptides identified with MS/MS spectra can be assigned to more than one protein, there is often some ambiguity as to which proteins were actually present in the sample. The guiding principle in this case is, in accordance with the Proteomic Standards Initiative (315), to create a minimal list of proteins, meaning the minimum number of proteins that can account for the observed peptides, also known as the principle of parsimony or Ockham's razor (242).

Figure 6.3 Protein inference problem.

Following database search proteins A, B and C are implicated by the presence of peptides 1, 2 and 3. The principle of minimal list of proteins (also known as principle of parsimony or Ockham's razor) should be applied in LC-based (shot-gun) proteomics. This means that the minimal number of proteins accounting for the present peptides should be reported. In the example, proteins A, B and C are present in a sample. Mass spectrometry analysis returns the sequence of three peptides present in the sample. According to the minimal protein list principle only A should be reported as A accounts for all three peptides, and there is no proof that either B or C was present in the sample. LC = liquid chromatography. See also Figure 4.3 on page 45.

The protein inference problem can be exemplified by (Figure 6.3). Imagine we have MS/MS spectra matching three peptides. These peptides are known to originate from three different proteins (A, B, and C) but does this mean we have evidence for all three proteins in our sample? In accordance with principle of parsimony, only A will be reported as present since it can account for the presence of all three peptides in the sample. Protein B and C will be reported as sub-set proteins, and given inferior status. This is a reasonable action but does not guarantee correct results. The sample could have contained a mixture of protein B and C but not A. Furthermore, if the match for peptide 2 is weak and deemed spurious, there is nothing dif-

ferentiating between protein A and B. This is the so called protein inference problem, and is of particular importance in LC-based proteomics where no protein separation is performed prior to enzymatic digestion, peptide analysis and quantification. In contrast, in gel-based proteomics the intensity of the separated protein spots is quantified prior to their digestion and identification. Compare this to figure Figure 4.3 on page 45.

In Paper II automatic algorithm based protein identification software were used such as SCAFFOLD and Protein Prophet, which contain probabilistic models for elimination of uncertain results. The validation with a decoy database resulted in a <1% decoy FDR. This means that the protein identification results are highly trustworthy, however, may have failed to identify proteins which were actually present in the samples.

The univariate multiple t-tests used in Paper II to identify statistically significant differences in protein quantity were employed without any correction for multiple testing, making these results more vulnerable to a type 1 statistical error. Corrections for multiple testing reduce the risk of a type 1 error, but in proteomics where the sample size is normally low ($n=10$ in Paper II), and the number of tests are in the hundreds, applying traditional methods, such as the Bonferroni method, would almost certainly result in a type 2 error, meaning failing to detect a difference which is present (316). Instead, to correct for a type 1 error the differentially expressed proteins can be placed in the context of pathways or biological processes, and more emphasis placed on the proteins in the top categories of such a network analysis. Thereby, false positives are assumed to be evenly distributed in groups but true changes should then cluster in the relevant processes (316). Another alternative to circumvent the problem of multiple univariate statistical tests is to apply multivariate regression modelling to describe structured variation in large and complex data sets.

7 CONCLUSION

A persistent metabolic disturbance indicating *mitochondrial dysfunction* was prevalent, despite *improved regional cerebral blood flow*, in the perihemorrhagic zone following surgery for ICH. This implicates mitochondrial dysfunction in the secondary injury processes of ICH, and presents a possible target for future interventions. Furthermore, proteomic analysis of protein expression, revealed *changes in the proteome of the perihemorrhagic zone over time* in comparison to in the seemingly normal, uninjured cortex. Significant pathways included inflammatory response, and regulation of endopeptidase and hydrolase activity. The results show that cerebral microdialysis (CMD) can be a useful technique for study of pathophysiological processes induced by ICH.

However, protein recovery was affected by *protein adsorption* to the CMD membranes. When CMD is used to sample and analyse macromolecules with a proteomic approach the membrane should be analysed in addition to the dialysate as protein adsorption could markedly lower protein concentration in the dialysate. Nevertheless, proteomic analysis of biomarkers following ICH sampled by CMD is feasible and presents a useful tool in the further exploration of the pathophysiological process of ICH.

The nation-wide retrospective study of surgically treated ICH patients in Sweden during a five year period revealed that *clinical features* of patients selected for surgery were similar across all six neurosurgical centres in Sweden. However, differences were found in ICH-specific characteristics such as *haematoma volume*, proportion of *deep-seated ICH*, and in aspects of *neurocritical care*. These differences reflect the lack of clinical guidelines for surgical and neurocritical care of ICH patients and indicate areas of focus for future research.

Apart from developing such clinical guidelines for ICH care, there is also a need for further research on reducing toxicity of and oxidative damage caused by the ICH, possibly by targeting mitochondrial dysfunction. Combining such novel medical treatments with less invasive surgical techniques may in the future reduce the high morbidity and mortality of ICH.

8 FUTURE DIRECTIONS

- In view of the suggested important role for neuroinflammation in the pathophysiology of ICH, temporal analysis of cytokines and chemokines sampled by cerebral microdialysis (CMD) in perihemorrhagic tissue (PHZ) and seemingly normal, uninjured cortex (SNX) will be performed.
- Given the suggested role of neurodegenerative long-term secondary injury processes following ICH, measurements of markers for axonal injury, synaptic activity and glial activation (A- β_{40} and A- β_{42} , NfL, t-tau, GFAP) in CMD-derived samples from PHZ and SNX will be performed.
- High resolution MRI will be used in subacute phase following ICH surgery to determine cerebral blood flow and tissue composition. Repeated MRI will be performed at long term follow-up to determine tissue outcome and chronic effects of ICH including neurodegeneration and atrophy.
- As near infra-red spectroscopy (NIRS) can be used to determine redox-state of tissue it may provide information on and even possibly improve mitochondrial dysfunction. Methods to employ this technique in ICH patients will be explored.
- In view of the lack of data on long-term functional outcome following surgery for ICH, the patient cohort in Paper IV will be evaluated to determine functional outcome (mRS) and quality of life (EQ-5D) at long-term follow up.

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INDEX

- 30-day mortality, 3, 7, 36, 71, 80, 82
- albumin, 43, 46, 63, 79
- antiplatelet therapy, 6, 6, 16
- arteriosclerosis, 4
- ATACH-2, 15
- ATP production, 12, 13, 28, 78
- avoidable factors, 16
- BBB, 9, 10, 77, 78
- cellular respiration, 27
- cerebral amyloid angiopathy
 - CAA, 3, 4, 5, 14
- cerebral blood flow, 22
 - CBF, 9, 22, 29, 40-42, 52-54, 58-59, 73-74
- cerebral microdialysis. *Se* CMD
- CLEAR III, 20
- clinical equipoise*, 18, 19
- clinical presentation, 14
- CMD
 - basic principle, 23
 - convection, 23
 - diffusion, 23-25, 76, 77
 - insertion trauma, 78, 79
 - limit of detection, 43
 - low-molecular-weight, 27, 40, 43, 60, 62, 75, 83
 - normal baseline values, 27
 - relative recovery, 24, 25
 - tortuosity, 24-25, 75
- coagulopathy, 6, 16
- corticosteroids, 37
- craniotomy, 18
- CTP, 42
 - error, 42
- cytokines, 11
- DALY, 3
- decompressive hemicraniectomy, 21
- decoy database, 52
- deep-seated location, 36
- deferoxamine, 17
- DVT, 17
- electron transport chain, 12
- endoscopic surgery, 20
- epidemiology, 3
- EQ-5D, 7, 83, 89
- ethics, 35
- fingolimod*, 17
- glutamate, 13
- haematoma expansion, 6, 8, 14-16
- haemopexin, 11
- haptoglobin, 11, 67
- hypertension, 3
- ICES, 20, 81
- incidence, 3
- INTERACT2, 15
- lobar location, 36
- long-term outcome, 7
- LPR elevation
 - definition, 43
- macromolecules, 25, 29, 75, 87
- main causes of ICH, 5
- medical complications, 17
- microglia, 11
- minimally invasive surgery (MIS), 20-21
- minocycline*, 17
- MIS, 20-21, 81
- MISTIE, 20, 81
- mitochondria, 12
- mitochondrial dysfunction, 1, 12-14, 27, 31, 72, 73-75, 87, 89
 - treatment, 75
- mitochondrial permeability transition pore. *Se* MPTP
- modified Rankin Scale. *Se* mRS
- MPTP, 12, 13
- mRS, 7, 19, 36, 83, 91
- multimodal monitoring, 22, 23, 37, 80
- near infra-red spectroscopy, 75
- neurocritical care, 22
- neurodegenerative, 14, 91
- neuroimaging, 6, 14
- neuroprotective, 7, 8, 10, 14, 17, 77, 81

- Neuro-QoL, 7
- NOAC, 6, 16
- oxidative phosphorylation, 11-13, 28, 29
- pathophysiology, 7
- patient reported outcome measures.
 Se PROM
- peptide mass fingerprinting, 48
- perihemorrhagic edema. *Se* PHE
- PHE, 5, 6, 8, 10, 17, 77
- posterior reversible encephalopathy syndrome, 5, 14
- PPAR- γ , 17
- primary brain injury, 7, 16
- principle of parsimony, 51
- PROM, 7, 83
- protein inference problem, 51, 85
- proteomics, 30, 43-45, 49, 62, 75, 84-86
 - gel-based, 30, 43-48, 52
 - LC-based, 30, 43-45, 49-52, 85
- QoL, 7, 83
- quality of life. *Se* QoL
- rCBF, 58-59, 62, 73
- reactive oxygen species. *Se* ROS
- regional cerebral blood flow. *Se* rCBF
- retrospective studies, 20
- ROS, 9, 11-13
- secondary brain injury, 1, 7, 9, 11, 15, 16, 22
- seizures, 6, 14, 16, 37, 57
- spot-sign, 6, 14
- STICH, 18-20
- STICH II, 19-20
- stroke unit, 15
- time to surgery, 57
- treatment guidelines, 14
- type 1 LPR, 27-29, 43, 61-62
- type 2 LPR, 27-29, 43, 61-62
- ventilator associated pneumonia, 17
- vitamin-K antagonists, 16
- Xe-CT, 40
 - error, 41
 - portable CT scanner, 41
- young patients, 3, 20

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