Characterising The Role Of Haptoglobin In Experimental Subarachnoid Haemorrhage

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Abstract

Subarachnoid haemorrhage (SAH) is a devastating event associated with significant mortality and morbidity. A large proportion of SAH patients either die or suffer permanent disability due to a delayed multifactorial injury processes involving blood vessels, diverse inflammatory processes and secondary injury mechanisms. Extracellular haemoglobin (Hb), released from lysed red blood cells after SAH, is thought to be one of the prime culprits that incite these pathological processes. The role of haptoglobin (Hp), a systemic acute phase protein and the primary Hb-scavenging molecule, has recently been postulated to play a role in the pathogenesis of cerebral arterial vasospasm and delayed neurological deterioration in patients suffering SAH. The aim of this project was to demonstrate the relationship between free Hb and Hp within the cerebrospinal fluid following SAH, using the previously validated rat filament model. The results show that whilst free Hb levels peaked at 24hr post-SAH, there was marked free Hb within the CSF as early as the 1hr post-SAH. In addition, there was an increase in CSF Hp and soluble CD163 macrophage haemoglobin scavenger receptor from baseline, concomitant with the Hb peak at the 24hr mark, with a steady and rapid taper off, in keeping with clearance of free Hb by 72hrs post-SAH. Additionally, histological assessment to examine macrophage receptor for uptake and subsequent degradation of the Hb/Hp complex showed sporadic parenchymal staining within the basal brain surface of rats that sustained SAH, but was absent in sham and control animals. This study adds further to the understanding of the way haemoglobin is handled in the central nervous system following SAH.

Declaration

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Author Contributions

The following people have contributed to authorship of the manuscripts enclosed in this thesis (in alphabetical order): Thomas C Morris, Renée J Turner, Robert Vink.

The individual contributions of each author can be summarised as:

Conceptualisation and documentation of the work: TM, RJT, RV.

Realisation of the work: TCM.

I give my consent for any manuscript(s) in which I am a co-author to be included in this thesis:

Thomas C Morris

Renée J Turner

Robert Vink

Publications

The following articles have been published, accepted or submitted for publication during the period of MPhil candidature, and sections of these articles are included in the present thesis.

Papers submitted for publication:

Morris T MBBS, Vink R PhD DSc, Turner R PhD. The Temporal Profile of Free Haemoglobin and Haptoglobin in a Rat Model of Subarachnoid Haemorrhage. Submitted for publication in *Neuroscience* November 2014

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Abbreviations

ABP – Arterial Blood Pressure

C – Centigrade

C1 – First Cervical Vertebra

CD163 – Cluster of Differentiation 163

CNS – Central Nervous System

CT – Computer Tomography

CSF – Cerebrospinal Fluid

HMG-CoA - 3-hydroxy-3-methyl-glutaryl-CoA reductase

DAB - 3,30 diaminobenzidine

DCI – Delayed Cerebral Ischaemia

DIND – Delayed Ischaemic Neurological Deficit

DND – Delayed Neurological Deterioration

ELISA – Enzyme Linked Immunosorbent Assay

Fe – Iron

Hb – Haemoglobin

Hb-Hp – Haemoglobin/Haptoglobin Complex

HO – Haem Oxygenase

Hp – Haptoglobin

ICA – Internal Carotid Artery

ICP – Intracranial Pressure

IgG – Immunoglobulin G

mm – Millimetres

mmHg – Millimetres of Mercury

NO – Nitrous Oxide

NHS – Normal Horse Serum

nm - Nanometre

PBS – Phosphate buffered saline

rpm – Revolutions Per Minute

SAH – Subarachnoid Haemorrhage

sCD163 – Soluble Cluster of Differentiation 163

SEM – Standard Error of the Mean

μm – Micrometre

μl - Microlitre

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Chapter 1
Introduction & Literature Review

1.1 Definition of Subarachnoid Haemorrhage

Subarachnoid haemorrhage (SAH) is the extravasation of blood into the subarachnoid space of the central nervous system (CNS), either over the surface of the brain, spinal cord or both (1).

1.2 Anatomy of the Subarachnoid Space

The coverings of the brain are composed of three distinct layers (Fig 1)(2). The tough fibrous outer layer, in contact with the structural supports for the neuraxis, is the dura mater. The next layer is the arachnoid, a translucent film that spans the cortical sulci and consists of web-like trabeculae that bridge the basal structures of the brain, creating a space known as the basal subarachnoid cisterns. The layer deep to the arachnoid is the delicate pia mater, which is invested in the brain itself. Over the convexity of the brain the pia is closely adherent to the arachnoid such that it is termed pia-arachnoid. Whilst the cortical subarachnoid space is essentially a potential space over the convexity of the hemispheres, at the basal surface of the brain the arachnoid is more closely related to the dura, which conforms to the shape of the underlying skull base. Here the subarachnoid space is comparably large and is filled with cerebrospinal fluid (CSF), as it exits the base of the brain through the two lateral and single median aperture(s) of the brainstem (3).

It is within these basal subarachnoid cisterns that the arteries supplying blood to the brain traverse. After entering the skull they join to form the anastomotic Circle of Willis, thence forming end arteries that supply terminal parts of the brain.

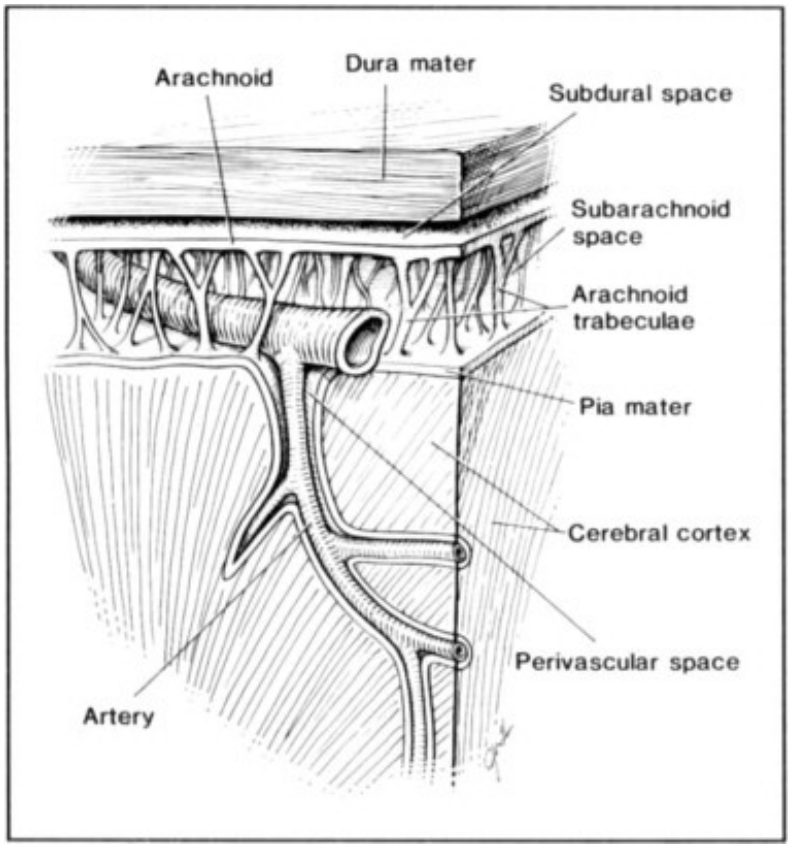

Fig 1. Schematic representation of the meninges and the subarachnoid space (2).

1.3 Aetiology of Subarachnoid Haemorrhage

Haemorrhage within the subarachnoid space has many causes. It may occur in isolation or in association with other kinds of haemorrhage, either subdural or intraparenchymal, depending on the inciting mechanism. Trauma, inflammation and infection can commonly cause a dif-

fuse, thin amount of subarachnoid blood to accumulate in a peripheral (more toward the vertex) distribution. Haemorrhage from a parenchymal brain tumour may lead to localised subarachnoid blood at the site of the bleeding, in addition to an associated intracerebral haematoma. The most common cause of significant SAH is that caused by a ruptured aneurysm arising from a cerebral artery, or less commonly a vascular malformation known as an arteriovenous malformation (AVM) (4).

Cerebral aneurysms, acquired outpouchings of arterial vessel walls, also known as saccular or "berry" aneurysms, tend to arise from branching points on the circle of Willis (Fig 2). Haemorrhage at these sites leads to a fairly classical appearance on computed tomography scanning (CT), with extensive clot filling the basal cisterns (Fig 3). This event, termed an aneurysmal subarachnoid haemorrhage, is a serious medical and surgical emergency that has significant sequelae (1).

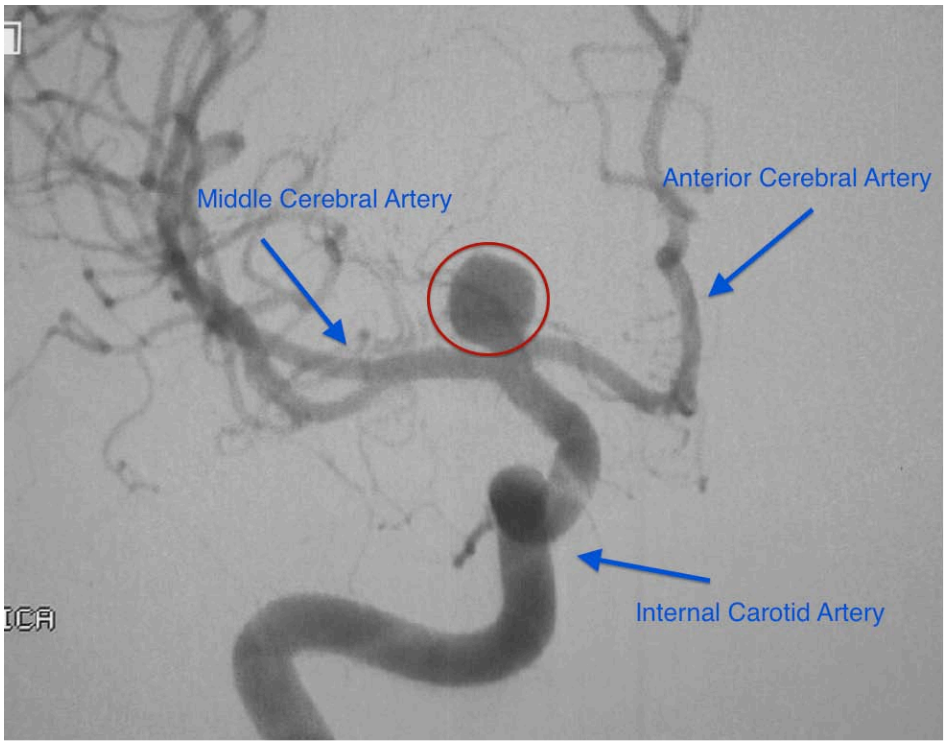

Fig 2. Catheter angiogram demonstrating saccular aneurysm (circled red) arising from the bifurcation of the right internal carotid artery. Personal image provided.

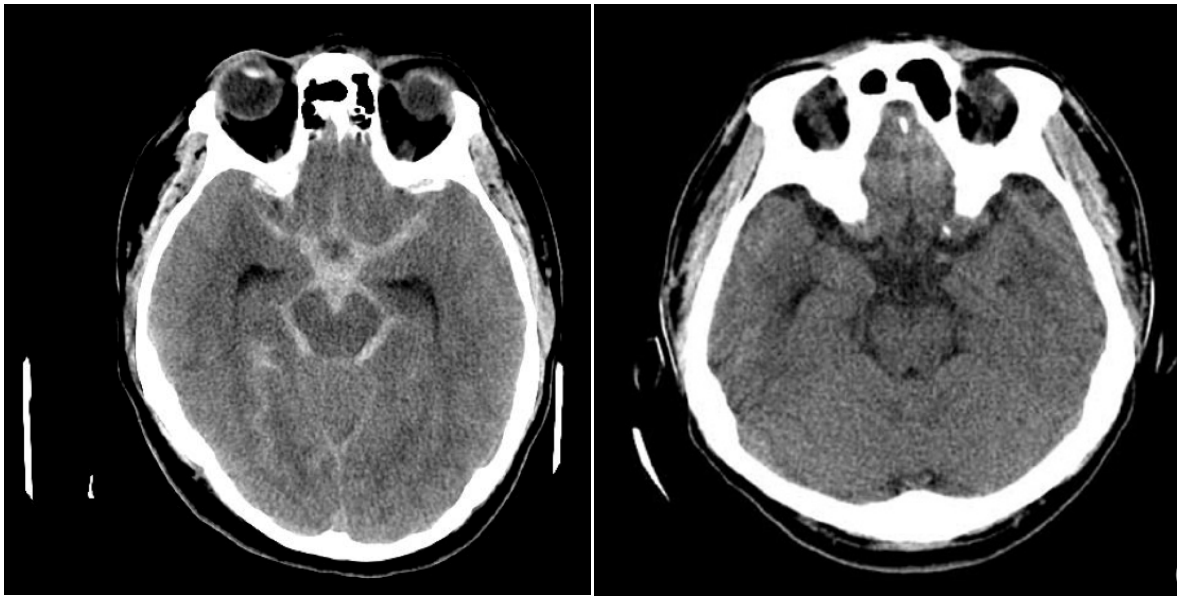

Fig 3. CT scans of the brain showing subarachnoid haemorrhage (left) versus normal findings (right). Personal images provided.

1.4 Epidemiology of Subarachnoid Haemorrhage

The crude incidence of SAH in Australia, not related to trauma or arteriovenous malformation, has been estimated at 10.3 cases per 100, 000 person-years (95% confidence interval [CI]: 10.2-10.4) (5). This equates to approximately 2,300 new cases of SAH per year in Australia. Females have a higher incidence of SAH (12.5 cases per 100,000; 95% CI: 12.3-12.8) compared to males (8.0 cases per 100,000; 95% CI: 7.8-8.3), with age-adjusted incidence increasing with age for both sexes. Less than 10% of SAH occurred in the first three decades of life. The peak age group for patients who experience SAH is between 45 years and 64 years, accounting for almost 45% of the overall annual SAH admissions (5).

1.5 Complications and Outcomes of Subarachnoid Haemorrhage

Aneurysmal subarachnoid haemorrhage has several serious complications. It may result in instant death (10-15%) if the haemorrhage is so severe that intracranial pressure (ICP) rises to a level such that cerebral blood flow ceases altogether. For those patients who survive long enough to receive medical attention, the chances of survival are still only in the order of ~70%, and nearly half of these patients will survive with significant disability (6). Obstruction of CSF pathways by clotted blood can lead to acute hydrocephalus, which may require shunt diversion to relieve elevated ICP. Haemorrhage from an aneurysm may result in intracerebral haematoma, in addition to the SAH, and this may precipitate brain herniation requiring emergent evacuation. Biochemical and hormonal alterations are common in patients suffering SAH, in addition to serious cardiac and pulmonary complications, meaning that the majority of acute SAH patients require admission to the intensive care unit and potentially have long stays in hospital (7). Strict control of blood pressure and fluid status are vital in these patients due to their precarious cerebral perfusion (4).

1.6 Management of Subarachnoid Haemorrhage

1.6.1 Surgical Management of Aneurysmal Subarachnoid Haemorrhage

For patients who have survived a SAH from an aneurysm there is the ongoing threat of re-bleeding until the aneurysm is completely secured. Re-bleeding is a major source of secondary morbidity and mortality in SAH patients; hence urgent treatment of ruptured aneurysms is the standard of treatment (8). Craniotomy (opening the skull) and micro-surgical exposure of the aneurysm may be performed, with specifically designed metallic aneurysm clips (Fig 4) applied in an attempt exclude the aneurysm from the cerebral circulation whilst preserving perfusion to the remainder of the brain (9)

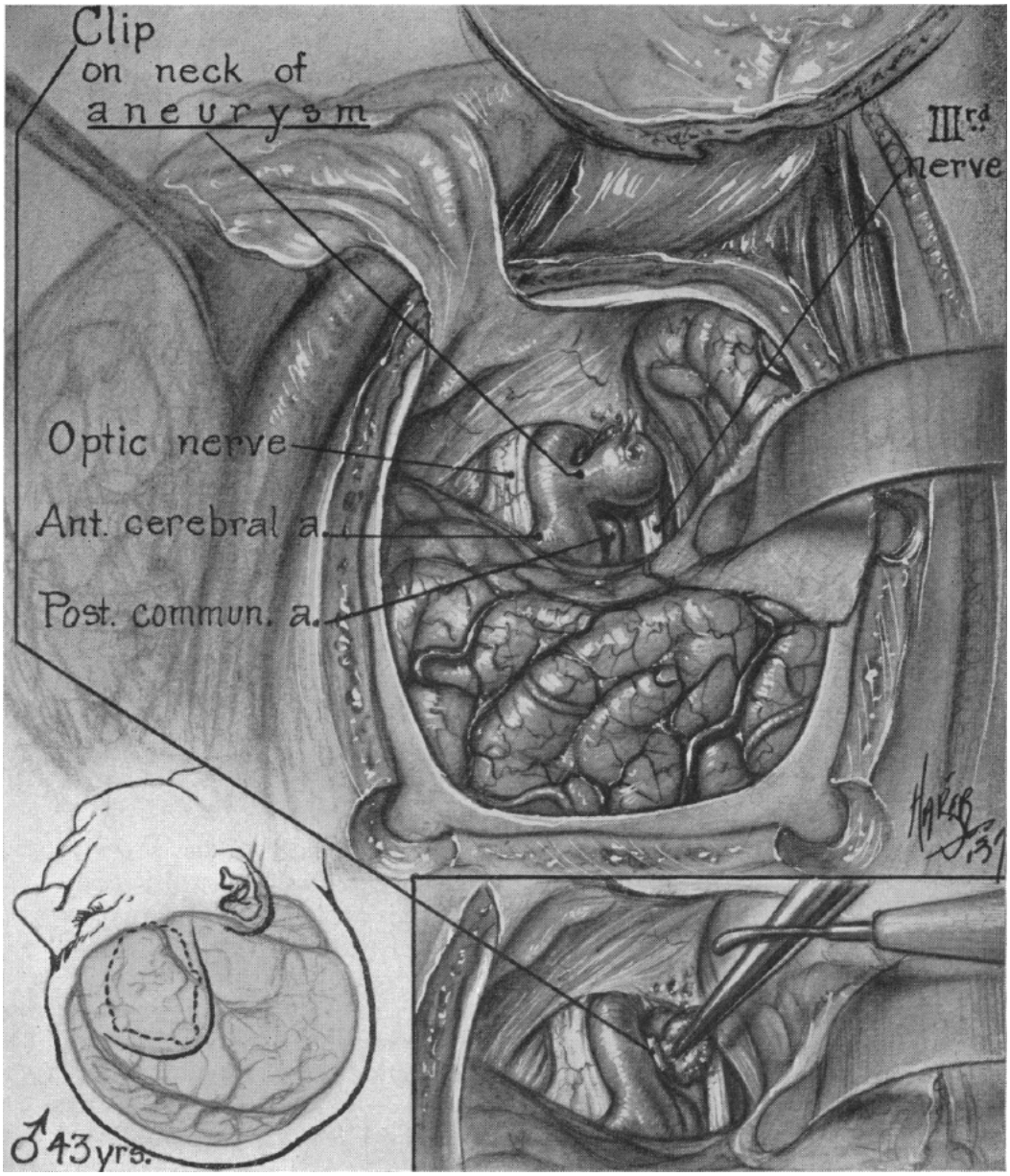

Fig 4. Original illustrative description of surgical clipping of cerebral aneurysm (9).

More recently, another technique for securing aneurysms has been developed. Endovascular deployment of tiny flexible metal coils, using a catheter from within the lumen of the originating cerebral artery, causes thrombosis within the fundus of the aneurysm, which also leads to exclusion from the circulation (10)(Fig 5). This technique has been shown to result in improved morbidity and mortality outcomes for patients compared to surgical clipping in patients who have suffered a SAH (11,12). However, there are concerns over the long-term du-

rability and efficacy of endovascular treatment of aneurysms and each patient's management is individually tailored (13,14).

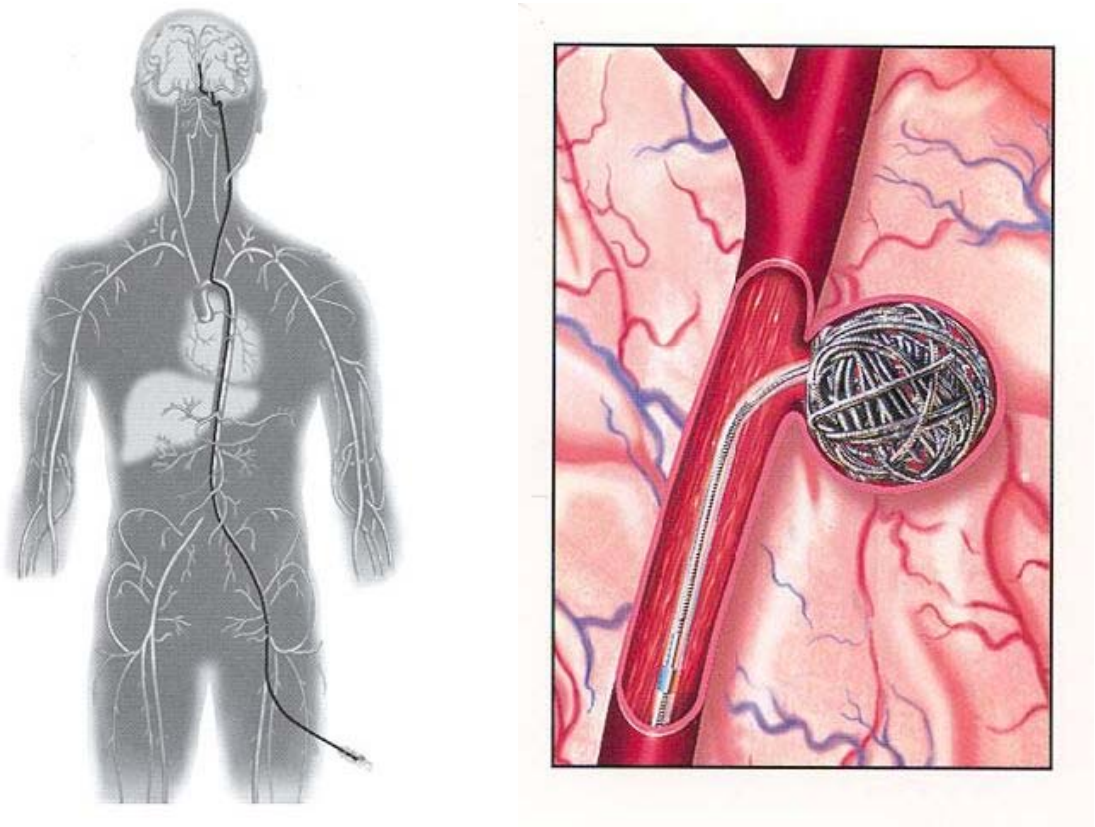

Fig 4. Schematic representation of endovascular coiling of cerebral aneurysm (15).

1.6.2 Medical Management

Patients who have suffered an aneurysmal SAH require close monitoring to detect deterioration from a multitude of complications that may arise (16). They need careful observation of vital parameters, blood chemistry and repeated physical examinations to assess for further deterioration and the need for possible intervention. In addition to repeated CT scanning to assess the evolution of the subarachnoid blood, circulation of CSF and to assess for further haemorrhage or brain injury such as ischaemia or infarction, patients may require lumbar punctures or external ventricular drains to deal with chronic hydrocephalus. Patients who survive their SAH sit on a spectrum of severity of brain injury, ranging from almost asymptomatic to completely vegetative. Patients almost invariably require a period of physical rehabilitation and a large proportion do not return to work (17).

1.7 Delayed Neurological Deterioration (DND)

The most common cause of poor outcome apart from the initial haemorrhage itself is an entity historically termed “vasospasm”, but probably more correctly known as delayed neurological deterioration. This clinicopathological entity is not restricted to aneurysmal SAH, and there is evidence of it occurring in the other causes of SAH as mentioned above, however by far and away its major significance lies with patients who have an aneurysmal source of bleeding.

In the days after an aneurysmal SAH, up to 30% of patients suffer from a clinical deterioration that cannot be attributed to any other complicating factor such as re-bleeding from an unsecured aneurysm, seizure, electrolyte disturbance, hydrocephalus or drugs/sedatives (18). Deterioration may manifest in a myriad of ways, ranging from subtle to severe motor and/or sensory deficits or from a mild decrease in the patient’s conscious state to profound coma and

eventually even death (approximately 7%). This cryptic worsening in clinical state occurs over a predictable time course in humans - commencing variably from day 4 post-haemorrhage, peaking at approximately day 7 and then abating from day 11. Histopathological examination of patients brains who succumb to this type of deterioration show widespread infarction of the brain, involving multiple arterial territories, including deep and cortical structures (19). This phenomenon was recognised early on in the history of the treatment of SAH, and its aetiology and pathophysiology has been researched extensively since.

1.7.1 Cerebral Vasospasm

It was also recognised early by clinicians, that concurrent with the clinical deterioration observed, that there was the development of significant cerebral arterial narrowing appreciated on cerebral angiograms performed on these patients. The onset of this radiographic abnormality, whilst being present in some patients from the initial onset of the SAH, seemed to correlate in severity with the clinical status of the patient, hence the term “symptomatic vasospasm”. A proportion of SAH patients (~40%), however, will have angiographic evidence of cerebral vasospasm without any clinical manifestations or ischaemic consequences (18).

This angiographic entity is not unique to SAH patients, and similar findings can been seen in other medical conditions affecting the cerebral vasculature such a cerebral arteritis and infections involving the central nervous system.

Histopathological examination of the affected arteries of patients suffering from vasospasm associated with SAH show non-specific chronic inflammatory changes. There is infiltration of the adventitia with inflammatory cells (lymphocytes, plasma cells and mast cells), myonecro-

sis and corrugation of elastin within the media, and smooth muscle hyperplasia, remodeling and opening of tight junctions of the intima (20–22). There is evidence of depletion of endothelial nitrous oxide, which is intimately involved in smooth muscle relaxation, and the vessels show hyper-reactivity with loss of their normal capacity for autoregulation (23), and up-regulation of vasoconstrictor receptors such as endothelin-1 (24).

The prevailing theory for much of the second half of the 20th century was that severe arterial narrowing, secondary to processes initiated by the SAH, leads to ischaemic insults due to cerebral perfusion deficits in the distribution of the affected vessels. The clinical manifestation of these perfusion deficits was termed delayed ischaemic neurological deterioration (DIND), but it has since become clear that not all patients who have demonstrable arterial narrowing (on catheter angiography or trans-cranial Doppler studies) have a clinical deficit and paradoxically patients may develop neurological deficits in the frank absence of demonstrable vessel spasm (25,26).

1.7.2 Delayed Ischaemic Neurological Deterioration

Whilst 70% of SAH patients show arterial narrowing, only half of these patients will manifest clinically. Furthermore up to 25% of patients will have magnetic resonance imaging (MRI) evidence of regions of cerebral infarction that remain clinically silent (27). Ischaemic stroke has been shown to be one of the most important factors in determining patient outcomes post-SAH and aggressive attempts to prevent stroke have become one of the mainstays of clinical management of DIND (28).

Attempts at predicting the onset of cerebral vasospasm and DIND lead to analysis of patient variables and the most consistent correlation was that of the absolute blood load in the basal

cisterns as seen on admission CT scans. Fisher et al in their oft-quoted paper described the predictable onset of vasospasm in patients who had significantly thick clots within the basal cisterns, as compared to patients who had minimal blood (29). This finding has been subsequently validated (30). Other variables have not been shown to be as consistently predictive, and this has added weight to theories of the pathophysiological mechanism involving the cisternal blood clot and/or its breakdown products after red blood cell lysis (31).

In addition to the human toll this disease takes, there is also the significant added financial aspect. Chou *et al* showed that the incremental financial cost attributable to radiographic vasospasm was 1.20 times higher (95% confidence interval, 1.06-1.36; P = .004) than for patients without vasospasm. Length of stay was an estimated 1.22 times longer for patients with radiographic vasospasm (95% CI, 1.07-1.39; P < .01). For symptomatic vasospasm, adjusted costs were 1.27 times higher (95% CI, 1.12-1.43; P < .001) and length of stay was an estimated 1.24 times longer (95% CI, 1.09-1.40; P < .01) for patients with vasospasm than for those without. The estimated cost in dollar terms for each patient with symptomatic vasospasm was calculated at an additional $US39,000 (32).

1.8 Current Management of Delayed Neurological Deterioration

1.8.1 Haemodynamic Management

Given that the prevailing theory behind patients' deterioration was that of cerebral vasospasm, and with no working pharmacological solution apparent in the early history of the disease,

initial therapeutic attempts consisted of trying to overcome the arterial stenosis physiologically. By augmenting the cardiovascular system to provide supra-normal blood pressure to the dysfunctional cerebral vasculature, the hope was to provide much needed perfusion to ischaemic regions. This treatment became the mainstay in neurosurgery critical care units around the world. Initially this treatment was known as “Triple H” therapy, which stood for Hypertension, “Hyperdynamic circulation” and Haemodilution (to improve blood rheology). This treatment had limited scientific foundation, but anecdotally was thought to be the best treatment available (33). More recently there has been a trend away from the use of hypervolaemia in the treatment of delayed neurological deterioration, with the focus being on euvolaemia and the use of vasopressor support to maintain supra-normal arterial blood pressure (27).

1.8.2 Mechanical Therapies

The use of balloon angioplasty to stenotic segments of proximally affected cerebral arteries has been shown to have some limited benefit, along with the use of intra-arterial vasodilators such as the calcium channel antagonists verapamil and nicardipine (27). Isolated reports of some success with irrigation of the subarachnoid spaces and removal of clot at the time of surgical clipping of aneurysms post-SAH has not been widely adopted, nor has continuous drainage of CSF in an attempt to remove products of clot lysis (34).

1.8.3 Pharmacological Management

The dihydropyridine calcium channel antagonist, nimodipine, is the only pharmacotherapy that has been shown to improve clinical outcome for patients suffering SAH (35). Meta-

analysis of the use of calcium channel antagonists (of which the trials using nimodipine gave the most statistical weight to) showed that their use reduces the risk of poor outcome and secondary ischaemia after aneurysmal SAH (36).

Other drugs that have been trialed include the statin class of cholesterol lowering HMG-CoA reductase inhibitors, various nitrous oxide donors, non-steroidal anti-inflammatory drugs, corticosteroids, antiplatelet drugs and endothelin receptor antagonists (37). Whilst pre-clinical and clinical evidence has shown the success of multiple agents in reducing the severity of arterial vasospasm, meta-analyses of these trials have consistently failed to demonstrate any beneficial effect on clinical outcome (38).

1.9 Angiographic Vasospasm and Delayed Neurological Deterioration - Current Theories

The repeated failure of agents whose primary effects relate to the amelioration of arterial vasospasm has lead to a wholesale change in direction of the research being undertaken (39–42). Whilst not completely ignoring the significance of proximal vessel spasm, focus has shifted to mechanisms relating to secondary brain injury attributable to the initial intra-cranial insult at the time of ictus (termed “early brain injury”) (43,44), and processes occurring in a delayed fashion well away from the proximal vessel spasm such as cortical spreading depression/depolarisations, microthromboses and distal microcirculatory dysfunction (45).

The concept of “spasminogens” relates to the elaboration of a soluble factor produced at the initiation of SAH that results in the delayed arterial narrowing seen at the time of clinical deterioration. Whilst research efforts to overcome the effects of arterial spasm may have appeared fruitless in light of the current evidence of a disconnect between arterial spasm and patient outcomes (as measured by performance indicators), a small body of literature does provide some insight into a possible link between the two (46). While it has been demonstrated conclusively that clazosentan (an endothelin-1 receptor antagonist) significantly improves vasospasm, it fails to have any significant effect on clinical outcomes (47–49). This result is perhaps best understood when assessing its effects in the pre-clinical literature. Clazosentan showed improvement in blood vessel caliber changes but failed to have any effect on the brain injury that most closely correlates with functional outcome, with no effect on neuronal cell death or focal cortical depolarisation (50). This contrasts with the demonstrated success of nimodipine, whilst having modest if any effect on arterial vasospasm, it has repeatedly shown to provide improvement in patient outcomes in human clinical trials, potentially through neuro-protective mechanisms (36).

Hence the search for a culprit turns to a substance that is capable of causing both the brain injury that is now thought to be predominantly responsible for poor patient outcomes, in addition to the arterial narrowing that coincides with this pathological process. Of course it is entirely plausible that these are two unrelated mechanisms, which are casually related. However, as stated in post-hoc analysis of the failed clazosentan trail, there was concern that the increased use of rescue angioplasty in the control arm may have been a confounding influence. This does lend weight to the idea that while arterial narrowing may not be the primary cause for clinical deterioration, it nonetheless may be one alterable factor in a multifactorial pathophysiological process (51).

1.10 Haemoglobin

Haemoglobin (Hb) is a protein synthesised within immature erythrocytes, with the primary function of oxygen transport (52). It is found in all vertebrates with several sub-types across species and within humans, which vary in their underlying genetic and peptide structure (53). In adult humans the vast majority of Hb is designated HbA, which is a tetramer globulin protein. Each of the four subunits are composed of a protein tightly associated with an iron molecule containing, non-protein haem group. The haem molecule is the site of oxygen binding, and oxyhaemoglobin is formed during cellular respiration when oxygen binds to the haem component of the haemoglobin after diffusion into red blood cells, and is transported around the body for supply to the tissues. Deoxygenated Hb is the form of Hb without the bound oxygen (54).

Hb breakdown normally occurs within the macrophages of the reticuloendothelial system in the liver and spleen, when erythrocytes near the end of their life cycle. The protein component is broken down and the amino acids are recycled (54). The rate-controlling step of haem breakdown is catalyzed by haem oxygenase (HO), which breaks down haem to form biliverdin, carbon monoxide, and iron molecules, which are biochemically recycled. In pathological states where haemolysis of red blood cells occurs, either within the circulation or other tissues, Hb may become free or extracellular and has many toxic properties (55,56).

1.11 Haemoglobin in Subarachnoid Haemorrhage

Given that Hb is the major constituent of red blood cells, this substance had been thought to be involved in the pathophysiology of cerebral vasospasm and DIND from an early stage. In-

deed, free Hb has been shown to exhibit toxicity through multiple mechanisms including the production of oxygen free radical molecules, lipid peroxidation, nitrous oxide (NO) scavenging, promotion of platelet aggregation and upregulation of blood vessel wall vasoconstrictor receptors (57,58).

In vitro and *in vivo* studies have demonstrated that free Hb in its oxygenated and deoxygenated forms contribute to the arteriopathy seen in aneurysmal SAH (59)and their concentration peaks coincide with the peak in clinical deterioration in the human condition (40). Tellingly, free Hb in the subarachnoid space has also been demonstrated to induce spreading depolarisation, leading to ischaemia and focal necrosis in the cortex of rats (60). Evidence of Hb acting simultaneously in both these pathological processes leads one to question the overall significance of Hb in the biochemical milieu of subarachnoid haemorrhage and delayed neurological deterioration.

1.12 Haptoglobin

Hp is a protein synthesised predominantly in the liver that is found in many animals including all mammals. Like Hb it is also a tetramer of protein subunits, whose main function is to bind free Hb. In humans there are three allelic subtypes, designated Hp(1-1), Hp(1-2) and Hp(2-2) (61). These subtypes have recently been shown to have specific implications in certain disease processes (62–64), including SAH (65,66). Patients who produce the Hp-2 subtype have been shown to suffer from a greater incidence of delayed neurological deterioration than patients of the Hp-1 genotype. This is thought to be due to their differing ability to effectively bind to and clear free Hb from the CNS (67).

Hb non-covalently binds to Hp with high affinity, forming a complex designated Hp-Hb. Its toxic effects are then minimized, however Hb's NO-scavenging ability remains even in this bound form (68). In disease processes where massive haemolysis and subsequent liberation of large amounts of free Hb may occur, and serum Hp levels can be undetectable. This is due to saturation of all available Hp molecules, which complex with Hb and are subsequently efficiently removed from the circulation by the reticuloendothelial system (69). Hp is also an acute phase protein, meaning its serum levels increase during periods of inflammation. Hp's role in inflammation has only recently been discovered and has widespread implications (70).

1.13 CD-163

Recently the cell membrane receptor CD-163 was discovered. This cell surface molecule has been shown to be expressed exclusively by monocytes and tissue macrophages, with distribution predominately in the liver and spleen (71). CD-163 positive macrophages have also been identified within the central nervous system, in the meninges, choroid plexus and perivascularly (72,73). The function of CD-163 was found to be specific binding of a neo-epitope of the Hp-Hb complex. This binding is high affinity, and was initially thought to bind only Hb in association with Hp, however subsequently it has been discovered that CD-163 also binds free Hb but with less affinity. This is thought to be a "back-up" process in the event that Hp is saturated, as this process occurs in a non-competitive fashion (74).

Subsequent to CD-163 receptor binding to the Hp-Hb complex there is endocytosis and internalization, with enzymatic breakdown of the Hb by haemoxygenase-1 (HO-1), and recycling of the Hp. The breakdown products of Hb such as carbon monoxide, bilirubin and Fe^{2+} have been shown to have anti-inflammatory properties, and more recent literature suggests that the CD-163 receptor may have intrinsic anti-inflammatory properties (75). In fact, the Hb-Hp-CD163 pathway has multiple cytoprotective anti-inflammatory properties, ranging from intravascular effects such as inhibition of the oxidation of haeme and prevention of the release of haeme from Hb, to cellular responses such as HO-1 upregulation (which has independent anti-inflammatory properties) and interleukin-10 synthesis (IL-10) (76). IL-10 promotes upregulation of CD-163 in a positive feedback loop, thus enhancing the capacity of the Hp-Hb-CD163 scavenging mechanism (74,77,78). Other effects of haeme metabolites generated by HO-1 activity include antagonism of pro-inflammatory cytokines, inhibition of platelet aggregation and vasodilatation (69).

In addition to the membrane bound receptor, a soluble form of CD-163 designated sCD-163 has also been characterised in serum and CSF. Increase in sCD-163 has been found to correlate with pro-inflammatory states compared to normal controls and it is thought to be a marker for monocyte/macrophage activity (79,80). This soluble form of CD-163 can also bind Hp-Hb and free Hb, but the purpose of this is yet to be elucidated. This process does not appear to affect the binding of membrane associated CD-163 to the Hp-Hb complex (81), and shedding of the receptor does is not thought to correlate with inhibition of the uptake of Hp-Hb complexes.

1.15 Haptoglobin-CD163 Scavenging System within the Central Nervous System

In their 2012 paper, Galea *et al* demonstrated just how overwhelmed the Hp-Hb-CD163 scavenging system within the central nervous system (CNS) is in the event of SAH (73). They compared CSF samples (taken from an external ventricular drain) of patients who had suffered a SAH (n=30), with control CSF specimens from patients undergoing lumbar puncture as an investigation for other reasons, which was subsequently found to be normal (n=20). They sub-stratified their cohorts according to their haptoglobin genotype (Hp-1 or Hp-2). Their findings were several-fold. Most significantly they showed that on average there was a 150-fold excess of free Hb to Hp, leaving the system completely saturated. There was indirect evidence of intra-thecal synthesis of sCD-163, using this as a proxy indication of recruitment of resident macrophages within the CNS. They reported that a relative hypohaptoglobinorrhachia (this neologism relates to a low concentration of haptoglobin within the cerebrospinal fluid) was seen in Hp-2 individuals and an absolute hypohaptoglobinorrhachia seen in the Hp-1 group. These findings were thought to be analogous to the effects seen in serum during episodes of intravascular haemolysis. Interestingly, however, there was no systemic increase in Hp levels (as would be expected during an acute phase of inflammation).

The group also found that Hp genotype influenced the likelihood of what they termed “delayed cerebral ischaemia” (DCI), their definition of which consisted of both clinical and/or radiographic findings, which was presumed to be attributable to cerebral ischaemia. The Hp-1 genotype appeared to confer some degree of protection from the effects of, at the very least, cerebral vasospasm and possibly DND. This finding is consistent with the findings of others (65,67). In fact, no patient with the Hp-1 genotype suffered DCI in their study. This was at-

tributed to a presumed superior ability of the smaller Hp-1 dimers to clear Hb and undergo endocytosis due to its structural difference to Hp-2, however this is at odds with the findings of Kristiansen *et al* who first described the CD-163 receptor, who showed that CD-163 has a 10 fold great avidity for binding to the multimeric Hp2-Hb complexes (79).

Curiously, Galea *et al* noted that unbound Hp was still detectable in the CSF of SAH patients, despite there being ample free Hb to bind to. This is in direct contrast to what is seen in the serum, where one would expect to find ahaptoglobinaemia in the presence of excess free Hb. They rationalised this finding as further evidence for "saturation of the system", which seems physiologically contradictory. Giving weight to this theory is the significant paucity of CD-163 macrophages within the CNS to bind and absorb any Hp-Hb complexes (indirectly evidenced by the 1200 fold difference between the serum and CSF levels of sCD-163). Levels from SAH patients showed a meager 6-fold increase in sCD-163 in the CSF, possibly indicating a degree of upregulation and/or recruitment of macrophages to the CNS, although this was not proven directly.

Several points should to be made regarding Galea *et al's* study. Firstly, the timing of CSF sampling was at an average of 3.9 +/- 2.6 days. A single sample, whilst giving a very broad appreciation for the fact that the Hb-Hp-CD163 scavenging system is inadequately equipped to deal with the massive influx of free Hb in SAH, does not give a true temporal representation of the evolution of what is occurring in the subarachnoid space. This is key given our current understanding of the pathophysiology of DND and cerebral vasospasm and the competing theories of early brain injury versus the contribution of released soluble factors etc. Yes, there is evidence that at a certain time point there is excess free Hb, however as we have seen with cerebral vasospasm, a casual relationship can be misleading. Several assumptions are made without direct observation and this has significant implications for our understanding of the disease, such as the recruitment of macrophages and upregulation of expressed CD-163 in the CNS. Also, the location of CSF sampling in the SAH group may not give a true represen-

tation of the composition of CSF at the site of haemorrhage, i.e. the basal cisterns. CSF flow traditionally has been thought to commence within the ventricular system and thence exiting the brain to be re-absorbed over the cortical convexities, which may mean that the sample was not taken from a location where one would expect the highest levels of any of the substances in question.

There have been no other studies looking at the role of Hp in SAH apart from Nonaka *et al*, who performed a small cohort study in patients who received an unspecified dose of Hp, delivered into the subarachnoid space at the time of surgical clipping of their cerebral aneurysm. Their conclusion was only that "the beneficial effects of Hp were noteworthy" (82).

1.16 Animal Models of SAH, Cerebral Vasospasm and DND

The use of animal models of SAH has been reviewed previously, and it is noted that the focus of most groups had been specifically on delayed cerebral arterial vasospasm. The disease has been studied across 7 species, with an excess of 65 models in the literature (83,84). The use of the rat model has consisted of several variations of a subarachnoid intra-cisternal injection of blood, and other methods involving the creation of haemorrhage within the subarachnoid space via perforation of a cerebral vessel at the base of the brain, with extravasation of blood under arterial pressure (85–91). Again giving reference to a more modern appreciation for the pathophysiological theories involved in delayed neurological deterioration, the two models were compared by Lee et al. Their conclusions were that the perforation model produced more severe pathophysiological changes than the blood injection model, and that the puncture model more closely mimics human SAH in having an injured blood vessel with a direct hemorrhagic brain lesion produced under arterial blood pressure, the extent of which is under the

control of normal physiological mechanisms. Therefore, their conclusion was that endovascular perforation seems a more suitable model for study of acute SAH sequelae (92).

1.17 Summary

Subarachnoid haemorrhage is a common and devastating disease, which strikes people in the prime of their lives. It is associated with significant morbidity and mortality, due to multiple sequelae from the initial brain haemorrhage. Delayed neurological deterioration is a poorly understood entity that remains a significant cause of morbidity and mortality in patients surviving a SAH. Apart from nimodipine, which has modest effects, there are no proven therapies for this disease process. The link between cerebral vasospasm and delayed neurological deterioration (and its presumed ischaemic equivalents) is also poorly understood. Successful treatment of cerebral arterial vasospasm has not resulted in improved clinical outcomes and there has been a shift in the nature of our understanding of the pathophysiological processes that follow SAH.

The role of Hb in delayed neurological deterioration and cerebral vasospasm has been suspected for some time. The detrimental effects on cerebral blood vessels and also its ability to cause brain injury, akin to that associated with delayed neurological deterioration have been demonstrated *in vivo*. Free Hb within the CSF is toxic. Hp, along with CD-163+ve monocyte/macrophages, is the body's natural defense against the toxic effects of free Hb, and it has been demonstrated that this defense is grossly inadequate within the CNS.

1.18 Hypothesis and Study Aim

The stated hypothesis is that haptoglobin plays a vital role in the pathophysiology of SAH, arterial spasm and delayed neurological deterioration.

This study aims to characterise the temporal profile of extra-cellular Hb in relation to Hp and the Hb:Hp receptor molecule CD163 in SAH, so as to gain a better understanding of the relationship between Hb breakdown products and the Hp/CD163 scavenging system within the CNS, and their role in cerebral vasospasm and delayed neurological deterioration after SAH.

Furthermore, if haptoglobin is demonstrably deficient then I hypothesize that the augmentation of haptoglobin will result in greater clearance of Hb from the CNS.

To the best of my knowledge, this has not been shown in humans nor in any animal models of SAH. A rat model of SAH, using the “filament” puncture technique has been selected to investigate this hypothesis.

Chapter 2
Methods and Troubleshooting

Methods

2.1 Animal Care & Ethics Approval

Animals (Sprague-Dawley Rat*)* were obtained approximately 1 week before the procedure to allow habituation to handling. They were group housed with unrestricted access to food and water. Ethics approval was granted by the University of Adelaide Animal Ethics Committee, approval number M-65-13. All experiments were conducted according to guidelines established for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council (8th Edition 2013).

2.2 Experimental Procedures

2.2.1 Experimental Design

The primary intention of the study was to quantify the CSF levels of free Hb, Hp and sCD163 in SAH, to see what relationship (if any) existed. Animals were randomized into naïve (n=5), sham (n=10) and SAH (n=20) surgery groups. Animals in the SAH group were further divided into sub-groups of 1hr, 24hr, 48hr and 72hr survival time-points (n=5/ time-point) after initiation of SAH. Sham animals underwent all invasive monitoring and surgical procedures apart from the actual initiation of SAH, while naïve animals underwent none of these procedures apart from general anaesthesia via endotracheal intubation and sampling of CSF via surgical exposure of the posterior atlanto-occipital membrane. The reason for having a sham

and naïve cohort was twofold – firstly, there was concern that placement of the burr hole and insertion of the ICP monitor could lead to spuriously elevated levels of free Hb in the CSF (this was not the case, see results in Chapter 3). Secondly, Hp is an acute phase protein, thus the trauma of the experimental procedures could have independently caused an increase in serum (and possibly CSF) levels of Hp. Thus, the naïve cohort provided previously unknown information regarding the normal CSF levels of Hp in rats.

The sequence of procedures was as follows: induction of anaesthesia, insertion of subdural ICP monitor, insertion of femoral arterial catheter, exposure of carotid and initiation of SAH, exposure of posterior atlanto-occipital membrane and sampling of CSF, perfusion-fixation and decapitation.

2.2.2 Anaesthesia

On the day of surgery animals were anaesthetised with isoflurane (Lyppard, Australia), administered by placing animals in a transparent induction box and delivering 3-5% isoflurane in oxygen 1.2 L/min. Once a surgical level of anaesthesia was reached, the animals were removed from the box, underwent endotracheal intubated and mechanically ventilated using a rodent ventilator. They were then placed on a thermostatically controlled heat pad to maintain normal body temperature throughout the procedure. Anaesthesia was maintained with Isoflurane 1.5-1.8% anaesthetic in oxygen at a flow rate of 1.2 L/min. Animals were ventilated at a rate of 70-90 strokes per minute with adjustments to ventilation parameters made according to results obtained from arterial blood gas analyses.

2.2.3 Placement of Intracranial Pressure Monitoring Device

Confirmation of the initiation of SAH was sought using readings from a fibre optic ICP-transducing device. Once general anaesthesia was established the animals were placed prone and the hair on the nape and head was removed. Alcohol skin preparation was applied. A small (~2mm) burr hole was placed in the right parietal bone, just behind the coronal suture. Two-point calibration of the probe was performed. A durotomy (cutting open the dural lining of the brain) was performed under microscopic visualisation, and the probe was tunnelled under the skin to secure it. The tip of the probe was advanced ~5mm within the subdural space under direct visualisation (Fig 6). Confirmation of correct placement was seen with a level ICP trace of approximately 10mmHg (Fig 7). The small incision was closed with surgical clips.

Fig 6: Right parietal burr hole placement (blue circle) and tunnelled sub-dural ICP monitor.

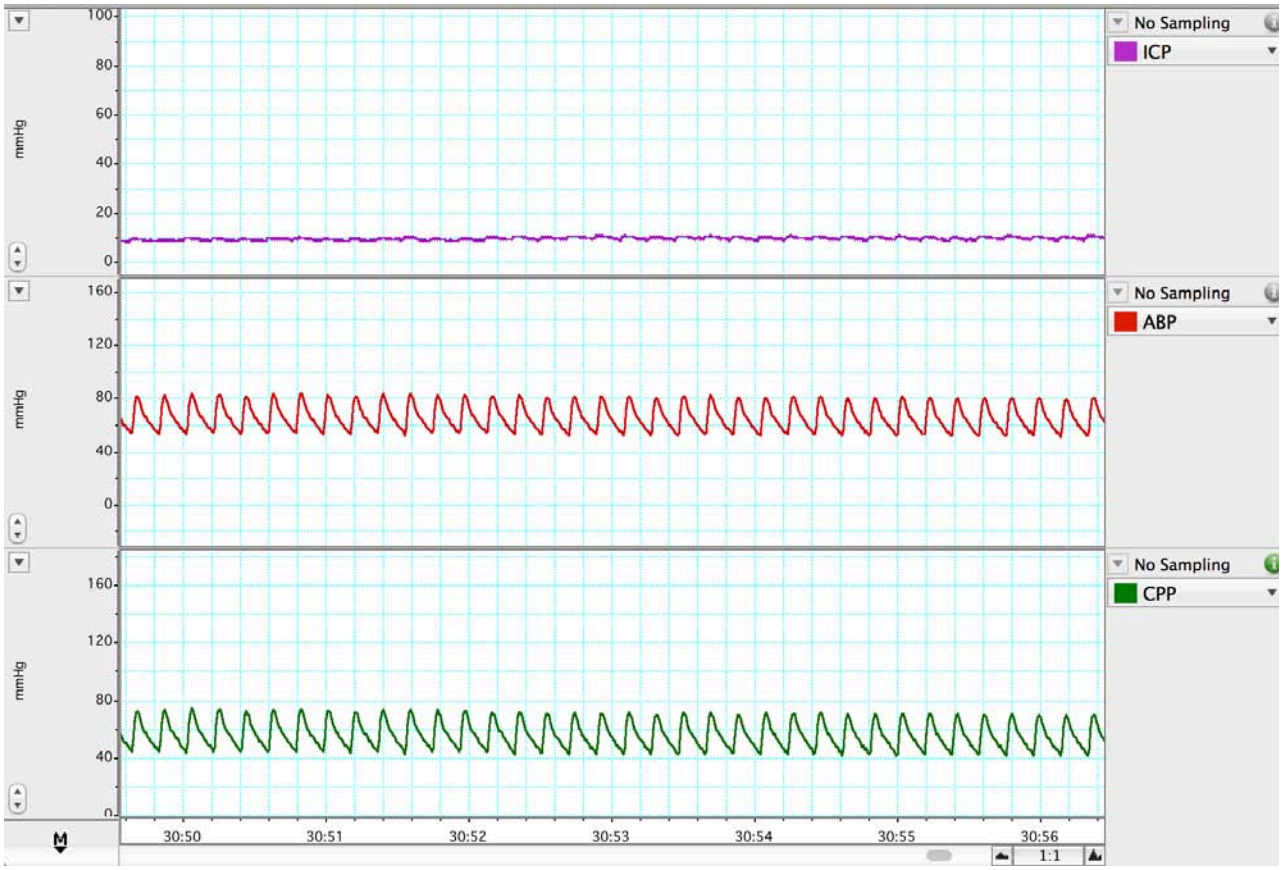

Fig 7: Baseline telemetry reading of ICP (purple), ABP (red) and real-time calculated cerebral perfusion pressure (green).

2.2.4 Femoral Arterial Catheterisation

Arterial blood pressure (ABP) was continuously monitored whilst animals remained under general anaesthesia. The animals were placed supine and the left groin was shaved and prepared with 70% alcohol. The left femoral artery was exposed and isolated. An arteriotomy was performed and a calibrated fluid-filled column attached to a pressure transducing monitoring device. Appropriate placement was confirmed with the observation of an arterial waveform. An arterial blood sample was taken as soon as the arterial line was inserted and blood

gas analysis was performed. Ventilatory parameters were altered in accordance with these results. Further blood gas analysis was performed throughout the monitoring period.

2.2.5 Initiation of SAH

The animals remained supine and hair of the neck and upper thorax was removed with clippers and prepared with alcohol. A midline incision was performed with exposure of the neck musculature. Retraction of sternomastoid and sternohyoid muscles with excision of the attachment of digastric from the hyoid bone led to adequate exposure of the common carotid artery on the animals left side. Isolation of the common, external and internal carotid arteries was undertaken using microsurgical techniques. A stump was made from the external carotid at its most distally accessible point (Fig 8). Temporary aneurysms clips were placed to trap the common carotid and internal carotid segments, so that arteriotomy of the external carotid artery could occur in a controlled fashion. A 4-O nylon monofilament was subsequently introduced into the lumen of the internal carotid, and was secured in place to prevent bleeding from the external carotid stump (Fig 9). The aneurysm clips were released and the filament was advanced to a predetermined length of ~20mm, at a point of appreciated resistance. Further advancement of the filament occurred in a controlled fashion using the intracranial pressure trace to confirm initiation of SAH (Fig 10). The filament was removed and the external carotid stump was tied off with nylon suture. Haemostasis was ensured and the wound was then closed with surgical clips. Anaesthesia was withdrawn once independent breathing was observed, the intubation tube was then removed and the animal was allowed to recover.

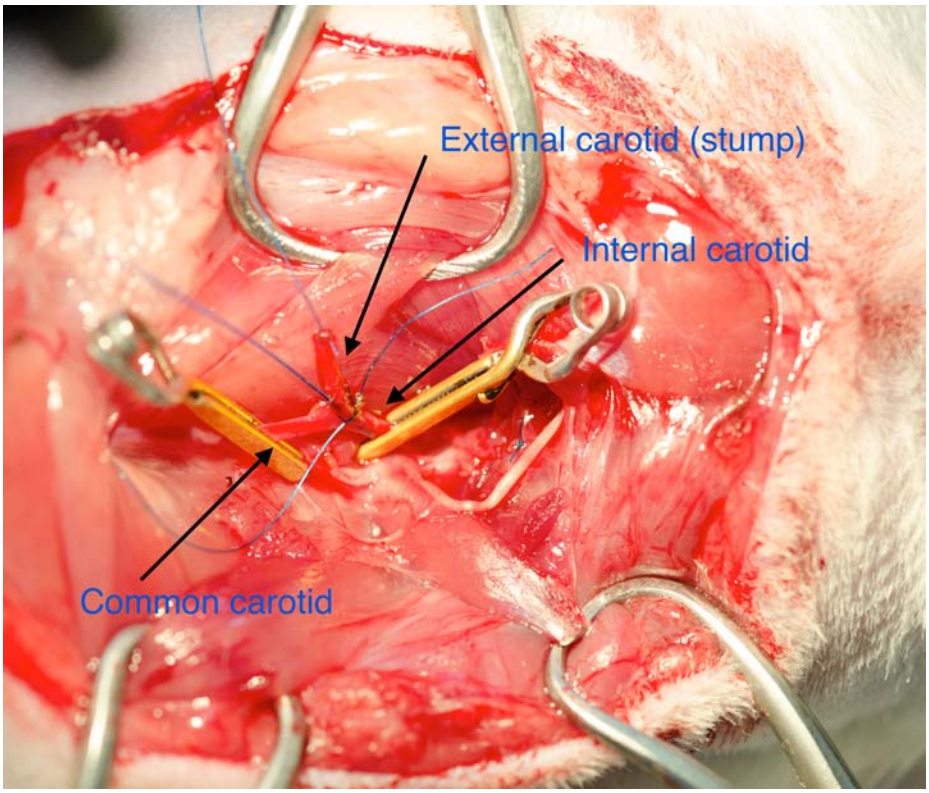

Fig 8: Surgical dissection of left common carotid artery, with isolation of external carotid (fashioned into a stump).

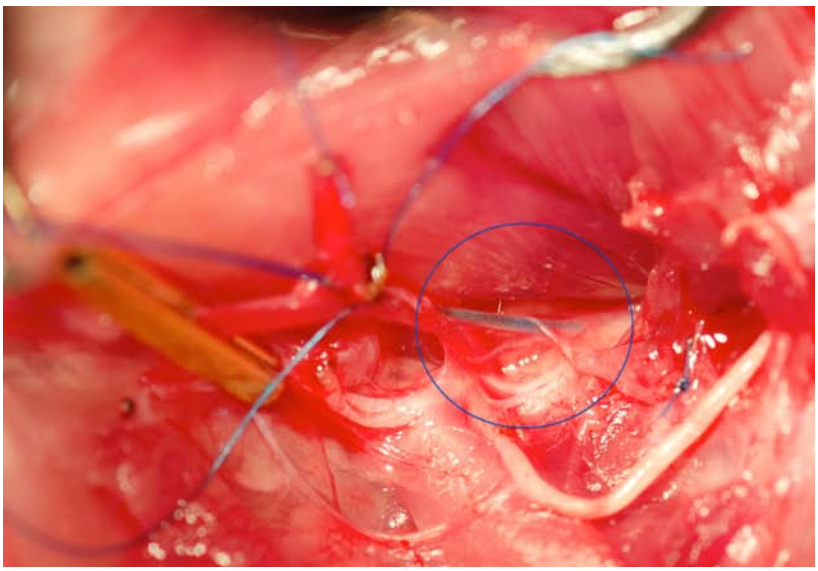

Fig 9. Filament seen passing within the lumen of the internal carotid artery (blue circle), about to enter the base of the skull, leading to the bifurcation into middle and anterior cerebral arteries.

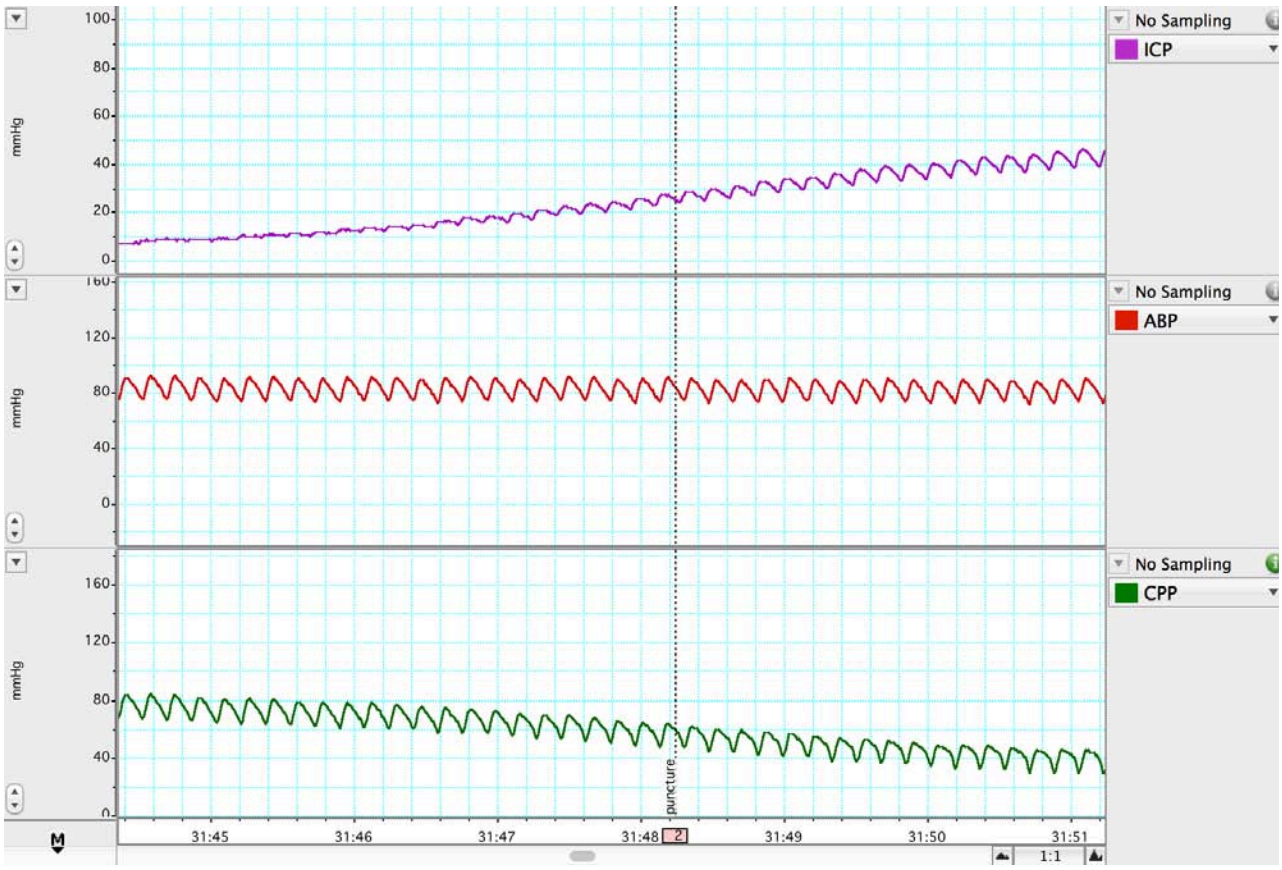

Fig 10: Screen capture at point of initiation of SAH. Time point marked as "puncture" indicates onset of dramatic rise in ICP (purple), a 6-fold increase from baseline moments before and an accompanying steep decline in CPP. The exact moment of puncture is approximately 3 seconds prior to that noted on the screenshot, as the operator was required to both perform the puncture and make the appropriate entry in the recording software. Note that arterial blood pressure remained unchanged during this period.

Animals were observed for at least 1 hr. after recovery from anaesthesia, or until they were conscious and ambulatory. At this point the animals did not require any specific post-operative care and were able to ambulate, self-groom and socialise at normal levels. They were then returned to their cage and given free access to food and water. Animals were checked several times per day to ensure no unexpected adverse effects occurred. Animals that demonstrated severe injury or signs of distress were immediately euthanized.

2.2.6 CSF Collection

At pre-determined time points (1, 24, 48, 72 hrs.), animals in SAH groups were re-anaesthetised and animals in the naïve or sham groups remained anaesthetised and secured in a prone position. The nape was shaved and prepared with alcohol and the skin infiltrated with local anaesthetic (~0.5ml of 1% lignocaine). A midline incision in the sub-occipital/nuchal region was then performed. Midline sub-periosteal dissection of nuchal musculature was performed with handheld diathermy with exposure of the sub-occipital region, the posterior C1 vertebrae and the posterior atlanto-occipital membrane (Fig 11). Under microscopic visualisation a 23-gauge needle attached to a 1ml syringe was used to puncture the posterior atlanto-occipital membrane, to a depth no more than the level of the bevel. Care was taken to ensure no inadvertent injury to any underlying vascular or neural structures occurred. This method of collection was adapted from those previously described (93–95), however given that it was imperative that no extraneous blood was sampled, the added surgical exposure was felt necessary to prevent accidental vessel puncture within the skin or muscles of the neck. Minimal vacuum was applied to withdraw cerebrospinal fluid, as to reduce any appreciable shear stress on red blood cells that may have been in the sample, as these forces may contribute to red cell lysis (96).

The specimens were immediately transferred into 1.6ml Eppendorph tubes and centrifuged at 1500rpm, at 4◦C for 10mins to separate any red cells from the CSF. The supernatant was carefully aliquoted off and placed in specially designed polyvinyl “cryovials” containers (Simport, Canada) suitable for storage of frozen specimens. The samples were snap frozen in liquid nitrogen and stored at -20°C for future batch analysis, which was deemed a safe procedure for the storage of the proteins of interest, namely Hb and Hp (97–99).

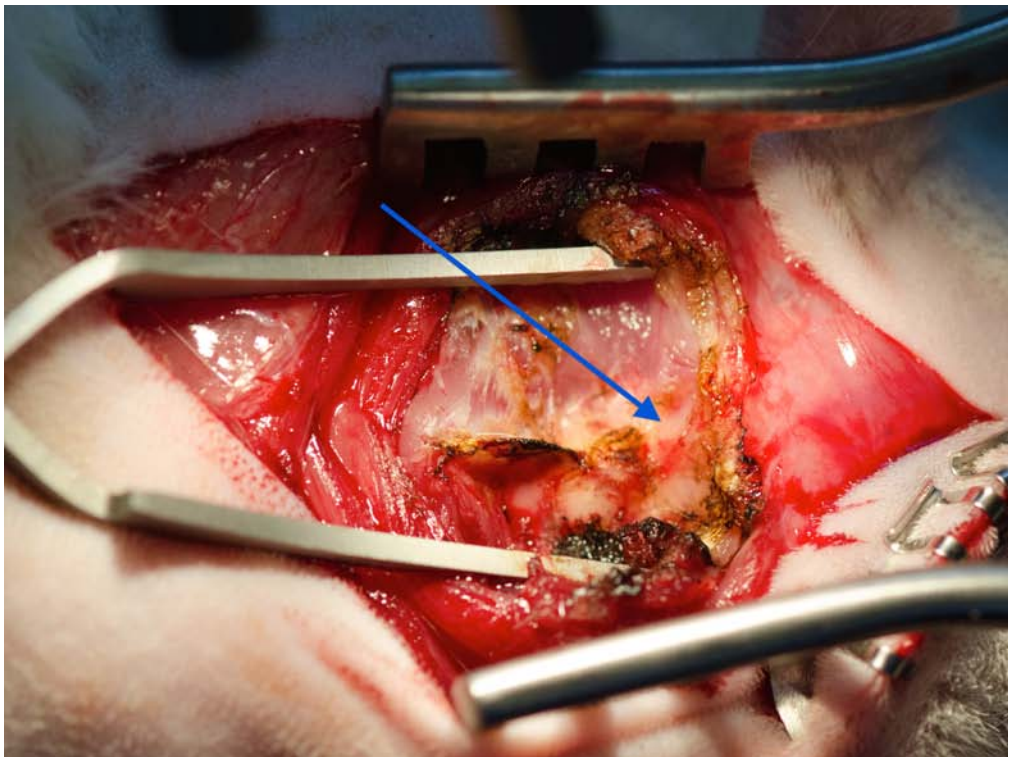

Fig 11: Surgical exposure of posterior atlanto-occipital membrane (blue arrow) for CSF sampling of cisterna magna.

2.2.7 Immunohistochemical Staining for CD163

After CSF was obtained the animals were perfuse-fixed and their brains were stained using a streptavidin-biotin peroxidase technique, performed on paraffin sections. Briefly, the sections were mounted on silane-coated slides and dried at 60°C for 20 mins. The slides were then de-waxed in xylene (3 changes of 2 min each) followed by absolute alcohol (3 changes of 2 min each). Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol at room temperature for 30 mins. The slides were then rinsed in phosphate buffered saline (PBS, pH 7.4, 2 changes of 3 mins). The slides were then placed in citrate buffer and placed in a microwave for 10 minutes at pre-determined appropriate power level, then removed and allowed to cool. The slides were then rinsed again in PBS buffer and the sections were then circled with a paraffin wax pen and incubated in 3% normal horse serum (NHS) for 30 mins. Slides were removed from the NHS and incubated with mouse monoclonal ED2 (CD163) (Serotec MCA342GA) at a concentration of 1/500 overnight at room temperature. The following day the slides were rinsed in PBS buffer again. They were then incubated with biotinylated anti-mouse secondary antibody (Vector BA-2000) for 30 mins. The slides were again rinsed in PBS buffer and then incubated with streptavidin peroxidase conjugate (ThermoScientific 21127) for 60 mins. Another rinse in PBS buffer was followed by the application of peroxidase substrate solution. This reaction was controlled visually to obtain optimum staining, for approximately 7 minutes. A final rinse in PBS buffer was followed by a wash in running tap water for 10 minutes. A counterstain was performed with Mayer's Haematoxylin. The slides were then dehydrated, cleared and mounted.

2.2.8 Colour Deconvolution Method

Immunohistochemistry uses specific inter-species antibodies to differentially stain tissue for antigens of choice. After application of a primary antibody, amplification can be detected with systems such as streptavidin-biotin coupled to 3,30 diaminobenzidine (DAB), which is precipitated to a brown reaction product in the presence of hydrogen peroxide. This technique relies on the principle that greater antigen content leads to increased precipitation of DAB. Analysis of immunohistochemical staining using DAB counterstained with hematoxylin can be combined with morphometric analysis of the chosen antigen in single brain regions or sub-regions (100). In this study we used this immunohistochemical method, with a mouse anti-rat CD163 monoclonal antibody as the primary antibody.

The use of color transformation techniques based on red-green-blue (RGB) intensity from 3-channel camera images can produce segmentation based on color, which can then be quantified (101). Mathematical methods for separation of the color information in RGB images have been developed, and this technique can be applied to a number of image types, including sections stained using immunohistochemical methods, which was used in the quantification of CD163 staining in this study.

The method we used, described in detail elsewhere, calculated the “dimensionless DABwt% value”, which provided a non-subjective estimation of the amount of antigen present. Moreover, using “virtual dissection”, the ability to analyze a specific region of interest, made it possible to estimate the antigen content in specific areas of the brain (100). Statistical analysis of these data (not shown) proved not to be significant however, and the gross inspection of the immunohistochemical preparations was felt to be more insightful (see results Chapter 3).

2.3 Model Difficulties & Troubleshooting

2.3.1 Mortality

The main hurdle encountered during the experiments was the unpredictability of animal deaths following the initiation of SAH. Whilst the mortality rate amongst the animals was within expected limits, the entire experiment (which consisted of several hours of preparation per animal) needed to be conducted prior to the initiation of SAH. If the animal promptly died then the opportunity to gain data was lost. There does not appear to be a way of modifying the model to reduce the level of mortality.

2.3.2 CSF Sampling

Whilst the model for producing the SAH is well established, the removal of CSF in sufficient quantity to be analysed was less straightforward. The added concern for this study was attempting to remove the CSF without contaminating it with systemic blood. Rather than stereotactic percutaneous sampling of CSF (93–95), it was felt that surgical exposure of the posterior atlanto-occipital membrane would be the safest way to ensure no spurious blood contaminated the samples. In addition to this, the CSF was centrifuged and the supernatant aliquoted away to ensure that the levels of Hb analysed were extra-cellular. This in itself was a concern, as the shear forces of a centrifuge could theoretically lead to damage to the red blood cells in the sample, thus care was taken to aspirate the CSF under minimal vacuum and the samples were centrifuged at particularly low speed (96).

2.3.3 Analysis of CSF Samples

Given the tiny volumes of CSF that were obtained, attempts were made to rationalise the way the analytes were tested for. Spectrophotometry is a well-established method for measuring Hb in a solution, with porphyrin molecules having a classic wavelength of maximum absorption called the Soret peak (around 400 nm, in the blue region of the visible spectrum) (102–109). A small body of literature also describes the use of spectrophotometry to quantify Hb and Hp, taking advantage of the fact that binding of Hb to Hp leads to structural change that is manifested in a shift in the Soret peak of the solution (110–114). A reducing agent (sodium metabisulphite, $Na_2S_2O_5$) can be added to the sample and a previously established mathematical equation allows for the quantification of free Hb, Hb bound to Hp and free Hp. Unfortunately, several attempts at replicating the spectrophotometric analysis of the Hb:Hp experiment failed to produce consistent results, however spectrophotometric quantification of Hb alone was easily reproduced in a reliable fashion. Thus it was concluded that an ELISA was the most suitable method for analysis of the CSF Hp and sCD163 levels, and spectrophotometry was used to quantify the Hb levels. Thankfully only tiny volumes were required for the commercially acquired ELISA kits so the average 100µl retrieved from each animal was adequate.

Although the ELISA is not able to differentiate between free and bound Hp, it was felt this was less important than the absolute quantities, as Hp has such high affinity for Hb that it can safely be assumed that whatever amount of measurable Hp present would almost certainly be in the bound form.

The commercially available rat Hp ELISA kit purchased called for an appropriate dilution of the sample such that the results would fall within the reference range of the provided standard curve. The original hypothesis was that the levels of Hp would be extremely low, especially

given the results of Galea et al (73), so the initial ELISA run used a conservative dilution factor of 1:50. Surprisingly this resulted in the majority of the results in the SAH specimens being well outside (above) the standard curve. This required another ELISA kit to be purchased and a re-estimated dilution factor of 1:200 was used, which provided results within the standard reference curve, allowing accurate calculation of the CSF Hp levels.

2.3.4 Augmentation of CSF Haptoglobin

One of this study's original intentions was to see whether augmenting the level of Hp (to the point of saturation) would have any impact (positive or negative) on any of the presumed correlates of poor outcomes in experimental SAH in rats (50). This was based on the assumption that there would be inadequate CSF Hp to deal with the amount of free Hb after SAH, as seen in humans.

The spectrophotometric analysis of Hb was performed prior to the ELISAs for Hp and sCD163 levels, demonstrating the peak of Hb as expected (see results in chapter 3). While waiting for the ELISA kits to arrive, some pilot experiments were performed in anticipation of the ELISA results. The plan was to inject rat Hp in solution (1mg/ml), directly into the subarachnoid space of experimental animals after the initiation of SAH.

Consideration was given to the appropriate anatomic location, and the method by which to deliver the Hp. Several key difficulties were considered. The volume of solution required to meet the stoichiometrically determined amount of Hp needed to saturate the expected amount of Hb in the CSF, in an average SAH, was found to be approximately 150µl. This volume ex-

ceeded the amount that could be administered via an intra-ventricular injection (at least if delivered as a single dose, the maximum volume for intra-ventricular injection being ~10µl). The suprachiasmatic cistern or the cisterna magna appeared to be the most suitable subarachnoid space to deliver this volume to, given they both have volumes approximating 150µl, and they are both used for injection of blood in established SAH models (85,92,115). However, after the initiation of SAH these spaces will immediately fill with clotted blood, thus essentially excluding the possibility of introducing any significant volume of Hp solution in a single injection, at least conceptually. Nonetheless a single attempt was made at a stereotactic injection of 150µl solution into the suprachiasmatic cistern of a rat immediately after the initiation of SAH. This resulted in rapid expulsion of the injected solution from the burr hole used to pass the needle.

Surprisingly the eventual Hp ELISA results rendered this hypothesis null and void (see results in chapter 3). Certainly if the Hp results had been as expected then perhaps a method of slow delivery via an implantable intra-cisternal catheter may have provided a solution to this problem. Additionally the original plan to perform various histological examinations on the experimental animals brains was abandoned due to this outcome, since in the end no therapeutic effect was tested for.

In summary, despite the steep learning curve of a technically demanding model and its associated high mortality rate, the stated hypothesis was able to be tested. Using this model and other described experimental procedures, meaningful results were obtained.

Chapter 3
The Temporal Profile of Free Haemoglobin and Haptoglobin in a Rat Model of Subarachnoid Haemorrhage

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Statement of Authorship:

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Dr. Renée Turner PhD – Oversight of experiments, data analysis and revision of manuscript.

Prof. Robert Vink DSc PhD - Oversight of experiments, data analysis and revision of manuscript.

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Abstract:

Background & Purpose: Aneurysmal subarachnoid haemorrhage (SAH) is a devastating event, associated with significant mortality and morbidity, a substantial proportion of which is attributable to events variously described as “symptomatic cerebral vasospasm” and delayed cerebral ischaemia. This pathological entity is thought to encompass multiple pathological processes involving blood vessels, inflammatory systems and secondary (“early”) brain injury mechanisms. The role of Hp, a systemic acute phase protein and the primary Hb-scavenging molecule in humans, has recently been postulated to have a role in the pathogenesis of vasospasm and delayed neurological deterioration in patients suffering subarachnoid haemorrhage.

Methods: We sought to demonstrate a temporal relationship between free Hb, Hp and the soluble form of the Hb scavenging receptor, sCD163, within the cerebrospinal fluid (CSF) in a filament model of SAH in male Sprague-Dawley rats. Hb levels were calculated using spectrophotometric analysis, Hp and sCD163 levels were assessed using enzyme-link immunosorbent assay (ELISA). In addition we performed immunohistochemical staining of the brain and meninges for the CD163 Hp/Hb complex macrophage surface receptor.

Results: This study has shown that whilst the peak in free Hb is at the 24hr mark, there is in fact marked free Hb within the CSF even at the 1hr post-SAH mark. In addition we found that, unlike other systemic haemolytic conditions, which see Hp levels drop, there is an increase in the measured levels of Hp in CSF, concomitant with the Hb peak at the 24hr mark. This is followed by a steady taper off, in concert with the clearance of free Hb by 72hrs.

Conclusions: Unlike human SAH, the rat appears to produce a robust Hb clearing response. There is clearance of Hb and Hp by 72hrs, which raises questions as to the role that Hp may play in human SAH. This study sheds further light on the way Hb is handled in the central nervous system after subarachnoid haemorrhage, and may point to future therapies for delayed neurological deterioration in these patients.

3.1 Introduction

Despite overall improvement in the mortality and morbidity of patients suffering aneurysmal subarachnoid haemorrhage (SAH) in the past few decades (116), the management of the syndrome of delayed deterioration after this event, nowadays most commonly referred to as "delayed cerebral ischaemia", has led to little in the way of improvement in outcomes for these patients. This pathological entity has been attributed to multifactorial processes involving blood vessels (23,45,48,117–119), inflammatory systems (120)and secondary (early) apoptotic brain injury mechanisms (45,121–124). Much work has gone into finding therapies that aim to ameliorate the impact of this condition, however currently there is little consistency in its management (125)and despite some promising results, no pharmacological cure (126).

The role of Hp, a systemic acute phase protein and the primary Hb-scavenging molecule in humans, has recently been postulated to have a role in the pathogenesis of cerebral arterial vasospasm and delayed neurological deterioration in patients suffering subarachnoid haemorrhage (127,128). Its role in other neurological diseases including intracerebral haemorrhage, traumatic brain injury and neuroinflammatory diseases, has been investigated (129–132). The role of Hb scavenging by Hp in subarachnoid haemorrhage and more specifically cerebral vasospasm and delayed neurological deterioration has only really been touched on in the past few years (67,73). In humans there are two allelic forms of Hp, and the different phenotypic subtype of Hp expressed has been shown to have an effect on the incidence of symptomatic cerebral vasospasm in humans (65,73,127,128).

There have been limited studies to date that demonstrate exactly what role Hp plays in the pathophysiology of SAH and delayed cerebral ischaemia. While Galea et al (73)have shown

that the Hb scavenging system is generally overwhelmed in SAH, no studies have documented the temporal relationship between the key components of this system in the event of SAH.

3.2 Materials & Methods

Studies were approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes (8th edition, 2013). Subarachnoid haemorrhage (SAH) was induced in Adult male Sprague-Dawley rats (n=56; 350-450g) which were obtained one week prior to surgery and were housed in a temperature-controlled conventional rodent room on a 12hr light-dark cycle with free access to food and water. Animals were randomized into naïve (n=5), sham (n=10) and SAH (n=20) surgery groups. Animals in the SAH group were further divided into sub-groups of 1hr, 24hr, 48hr and 72hr survival time-points (n=5/ time-point) after initiation of SAH.

3.2.1 Endovascular Perforation Model of SAH

The endovascular perforation model of SAH was selected as it most closely represents the human condition, with directed arterial rupture and the subsequent intracranial milieu resulting from dramatic intracranial and haemodynamic changes thereafter (92), as previously described (86). Briefly, animals were anaesthetized using 5% Isoflurane in an induction box with subsequent endotracheal intubation, mechanical ventilation (Harvard Apparatus, Holliston MA) and maintenance anaesthesia with 2% Isoflurane in a mixture of oxygen and room air. After placement of an intracranial pressure-monitoring device (described below), the left common carotid artery was isolated and the internal carotid artery (ICA) punctured at its bi-

furcation using a sharpened 4-O nylon monofilament. A dramatic, obvious and sustained rise in the ICP was used to confirm the initiation of SAH, in most instances the ICP rose to >50mmHg. Sham animals had advancement of the monofilament into the ICA but no puncture of the ICA, and no rise in ICP was seen in any of these animals. If an animal was randomized to the SAH group but did not have an indicative rise in ICP then this animal was excluded from the study.

3.2.2 Physiological Monitoring

Animals were kept at constant physiological temperature (37°C) using a rectal thermometer and thermostatically controlled heat mat. Upon initiation of anaesthesia all animals underwent right parietal burr hole and placement of a subdural fibre optic intracranial pressure-monitoring device (DePuy, Raynham MA). Animals then underwent catheterization of the left femoral artery for arterial blood gas sampling and continuous arterial pressure monitoring. Arterial blood pressure and ICP data were recorded continuously for the entire time that the animals remained under anaesthesia (LabChart 8 software and PowerLab data acquisition hardware, Colorado Springs CO).

3.2.3 CSF Collection and Analysis

At the pre-determined time-points, animals were re-anaesthetized (except the Sham, naive and 1hr cohorts which remained under anaesthesia) and underwent microsurgical exposure of the posterior atlanto-occipital membrane. Naïve animals had CSF sampling only, with no other procedure performed. Sham animals had CSF sampling done 1hr after sham puncture of the internal carotid artery. The SAH cohorts had CSF sampling done at the pre-determined time points after initiation of SAH. A 23G needle attached to a 1ml syringe was used to puncture

the membrane and remove approximately 100µl of CSF from the cisterna magna. Care was taken to minimize vacuum to reduce shear stress on red blood cells as the CSF was removed, and the depth of the needle was observed at all times under microscopic guidance. The CSF was subsequently centrifuged at 1500rpm, at 4°C for 10 minutes. The supernatant was aliquoted into polyethylene vials and snap frozen in liquid nitrogen and stored at -80°C for later analysis.

Spectrophotometric analysis of the CSF was done using a NanoPhotometer P-class spectrophotometer (Implen, Westlake Village, CA), with a path length of 1mm. A previously established algorithm using wavelengths of 380nm, 415nm and 450nm was used to determine the concentration of free (extra-cellular) Hb in CSF, measured in mg/100ml (107,133).

Analysis of CSF Hp was done using an ELISA kit according to the manufacturers instructions (Life Diagnostics Inc., West Chester PA). Briefly, the rat Hp ELISA is based on a solid phase enzyme- linked immunosorbent assay. The assay uses affinity purified anti-rat Hp antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-rat Hp antibodies for detection. The test samples were diluted to 1:100 with diluent provided, and incubated in microtiter wells for 45 minutes. The microtiter wells were subsequently washed and HRP conjugate was added and incubated for 30 minutes. The wells were then washed to remove unbound HRP-labelled antibodies and tetramethylbenzidine reagent was then added and incubated for 20 minutes at room temperature. This resulted in the development of a blue colour. Colour development was stopped by the addition of a stop solution, changing the colour to yellow, and optical density was measured spectrophotometrically at 450 nm. The concentration of Hp was proportional to the optical density of the test sample as

compared to the standard curve created concurrently with the test samples. Concentration of Hp was then calculated in mg/100ml after taking into account the dilution factor. The levels of soluble CD163 were obtained in a similar manner using a commercially available ELISA kit (MyBioSource Inc., San Diego CA) and expressed as ng/ml.

3.2.4 Histological Examination

Following CSF sampling, the animals were transcardially perfused-fixed with 10% buffered formalin. Brains were removed and placed in 10% buffered formalin solution for a minimum of 24hrs and 2mm coronal sections prepared using a rat brain-blocking device (Kopf Instruments, Tujunga CA). Brain slices were then processed, embedded in paraffin and 10μm sections cut. Sections were stained with hematoxylin and eosin, for light microscopic observation. For the immunostaining, 5μm sections were incubated with ED2 (1:800, MCA342R, AbDSerotec, Raleigh NC) a monoclonal mouse anti-rat CD163 receptor IgG1 antibody. Digital images of the prepared slides were created using a NanoZoomer whole-slide imaging device (NDP Scan U10074-01, Hamamatsu Photonics K.K,). Colour deconvolutional analysis of immunohistochemically stained slides was performed using previously described techniques (100).

3.2.5 Statistical Analysis

Data are expressed as a mean+/-SEM. Spectrophotometric analysis of free-Hb data and sCD163 ELISA were analysed by one-way ANOVA followed by Tukey post-hoc test. Hp ELISA data were analysed using one-way ANOVA and Sidak's multiple comparisons test. A *p* value of <0.05 was considered statistically significant. All statistical analyses were per-

formed using GraphPad Prism 6 for Mac.

3.3 Results

The perforation model of SAH in the rat has a well-documented mortality rate approaching 45% (92). This was our experience also, with 21 of 41 rats randomized to SAH cohorts dying prior to CSF sampling, at an average time of 8.6 hours post-SAH (range 0-44hrs). These animals were excluded from the study. A third (7/21) of these deaths resulted from immediate precipitous elevation in ICP >100mmHg and cardiac arrest whilst still under anaesthesia.

3.3.1 Quantification of Free Haemoglobin in CSF

Analysis of the spectrophotometric data produced a temporal profile of free Hb (Fig. 12). As indicated by low levels in the sham group, the CSF sampling procedure itself produced a negligible amount of measurable free Hb. A significant increase in free Hb was observed at 1hr ($p<0.05$) and 24hrs ($p<0.01$) post-SAH, with the 1hr peak reaching nearly 90% of the maximal peak seen at 24hrs post-SAH. By 48hrs free Hb levels had declined and were comparable to shams by 72hrs post-SAH.

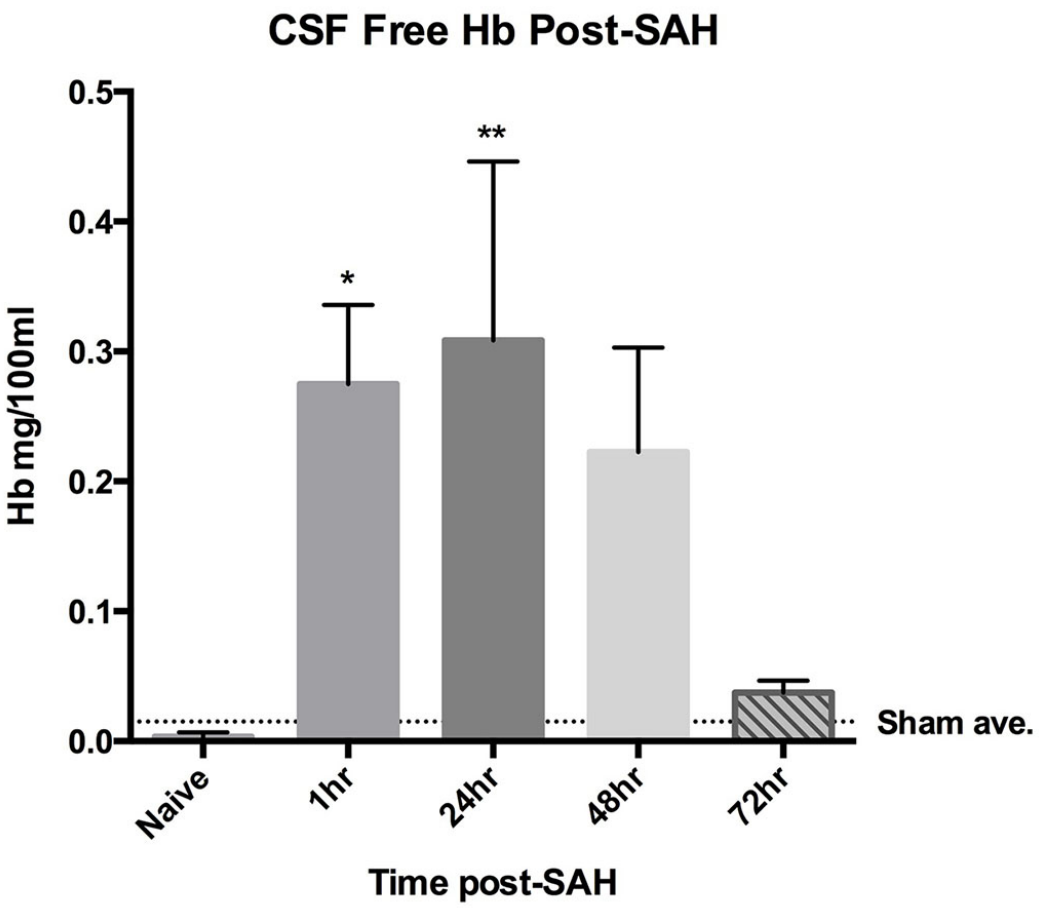

Fig 12. Spectrophotometric analysis of CSF free Hb following SAH (* denotes $p<0.05$, ** $p<0.01$ compared to sham).

3.3.2 Quantification of Haptoglobin in CSF

Rat Hp ELISA performed on the CSF produced a temporal profile of Hp (Fig. 13), showing detectable but low levels of Hp within the CSF in the naïve group. Contrary to predictions, there was a significant increase in Hp levels following SAH. At 1hr levels of Hp were comparable to the sham and naïve cohorts, but by 24hrs a significant ($p<0.0001$) rise in Hp levels was observed. This rise appeared briefly, tapering off in concert with the diminishing levels of free Hb (Fig. 12). By 72hrs post-SAH, Hp was approaching baseline levels, and this difference remained significant (72hr vs. 24hr $p<0.0001$).

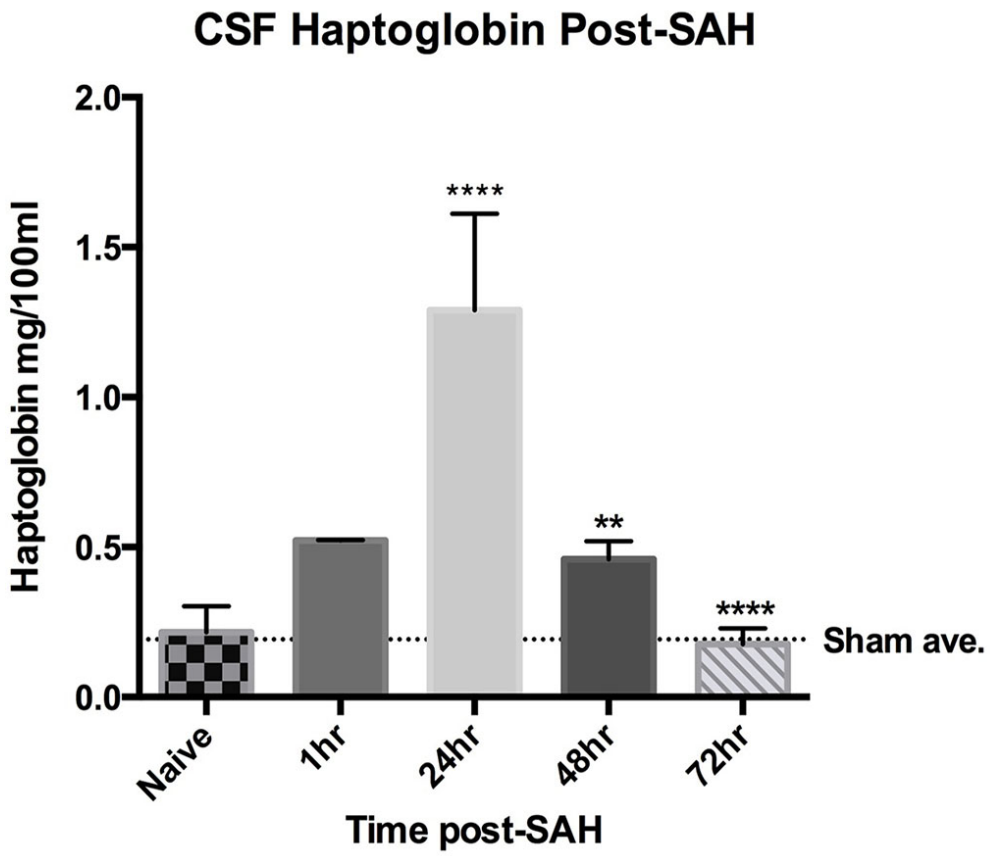

Fig 13. ELISA quantification of rat CSF Hp following SAH. Hp levels were significantly elevated at 24hrs post-SAH (sham vs. 24hrs, ****$p<0.0001$), which tapered off but remained higher than shams until 72hrs post-SAH. 24hr vs. 48hr (** $p<0.0031$), 24hr vs. 72hr (**** $p<0.0001$)

3.3.3 Quantification of Soluble CD163 in CSF

The levels of the soluble CD163 scavenger receptor (Fig 14), mirror that of Hp in a somewhat delayed fashion. By 24hrs there is a statistically significant rise ($p<0.01$), with the levels remaining elevated until clearance of Hb is achieved. As observed with Hp, levels of sCD163 returned to baseline levels by 72hrs post-SAH.

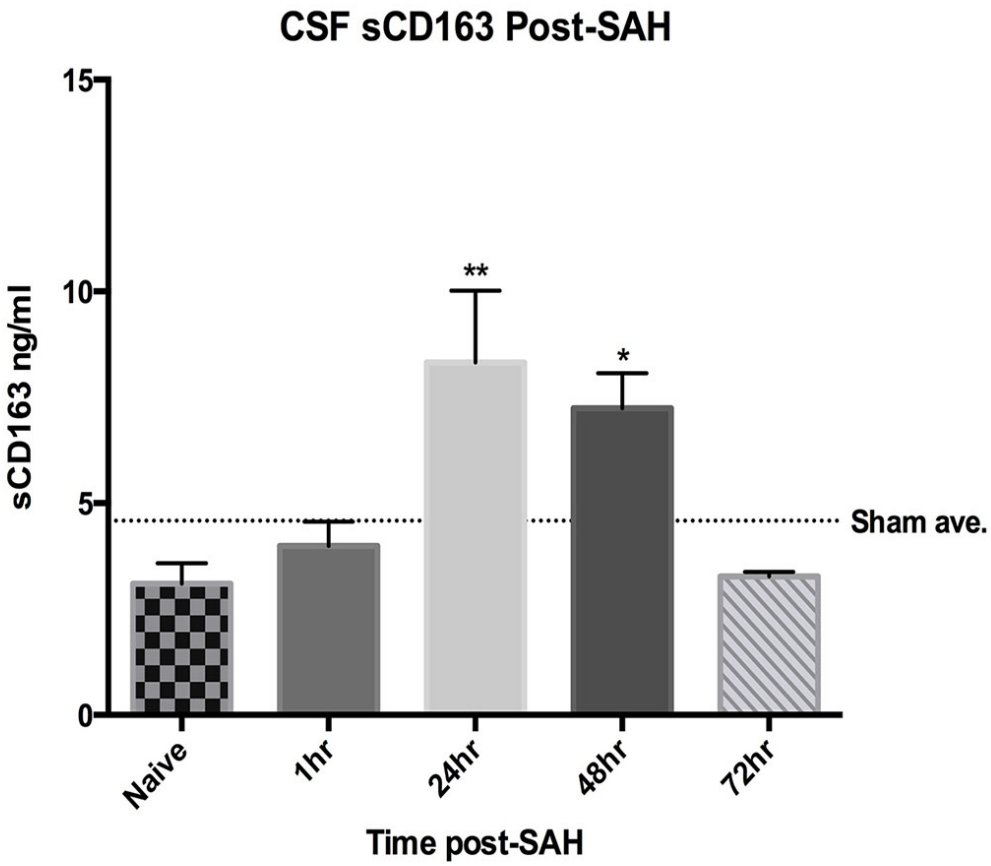

Fig 14. Rat sCD163 ELISA quantification of CSF. Significant results: sham vs. 24hr (**p<0.01), 48hr vs. 72hr (*p<0.05).

3.3.4 Immunohistochemical Staining for Haemoglobin/Haptoglobin Scavenging Receptor CD163

Immunostaining for CD163 showed great variability (Fig 15). Colour deconvolution analysis of these specimens did not demonstrate any significant difference between the level of CD163 immunoreactivity groups (data not shown). However, examination of individual sections revealed appreciable staining in the basal cortex of some animals that had experienced SAH, though not others. Whilst this was not readily quantifiable, the pattern of parenchymal staining was not seen in any animals within the sham or naïve groups. Staining in SAH animals was limited to the basal surface of the brain, with no clear relationship to the time after the

initiation of the haemorrhage, however the specimens with the most obvious staining occurred in the 48hr and 72hr animals Fig 15(C&D).

The areas which were seen to stain most consistently for ED2, were the basal cortex and brainstem structures. Macroscopic examination of specimens demonstrated most of the sub-arachnoid blood immediately subjacent to these areas. Further staining was seen in the cortex immediately underlying the site of insertion of the ICP probe. On higher magnification the staining appeared to be diffusely extracellular, within the substance of the parenchyma, perhaps reflecting the soluble molecule (sCD163). Although no co-localisation studies were performed, the staining was not seen within cells readily identifiable as phagocytes.

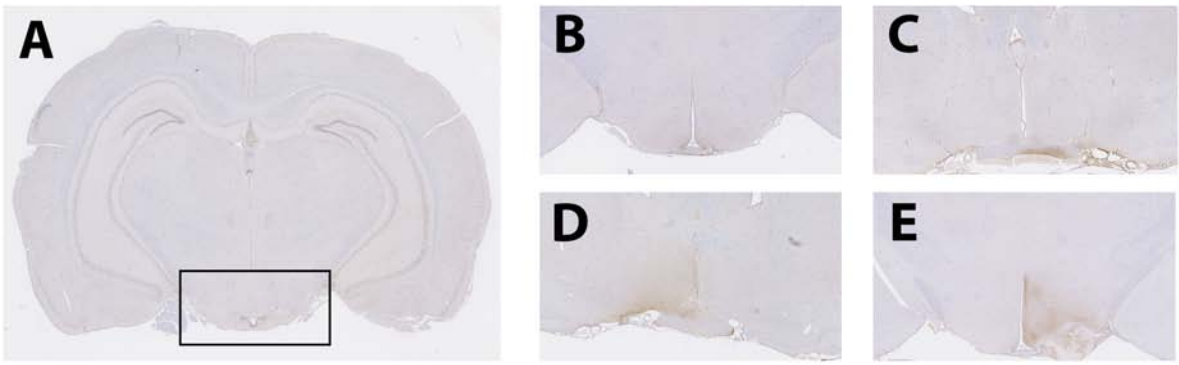

Fig 15: CD163 immunohistochemistry **(A)** Example of sham animal brain with box indicative of area of interest (40x). Sham animals **(B)** demonstrated negligible CD163 staining however increased immunoreactivity was observed at 1hr post-SAH (C) and was further increased at 48hrs **(D)** and 72hrs **(E)** following SAH. (Images B-E 80x magnification).

3.4 Discussion

This is the first study to describe a temporal profile demonstrating the relationship between Hb, Hp and sCD163 levels in SAH in any species, and the first to clearly show that Hp plays a significant role in the clearance of free Hb in the setting of SAH. The onset of SAH resulted in a rapid release of extra-cellular Hb into the CSF, beginning at the earliest sampling time-point of 1hr post-SAH. Free Hb peaked at the 24hr mark, which coincided with the peak in both Hp and sCD163 levels. As detectable levels of Hb returned to baseline by the 72hr mark, so too did the levels of Hp and sCD163, suggesting that this scavenging system is intimately involved in the removal of Hb in the setting of SAH.

The idea that free Hb acts as a toxin in the central nervous system is not new (134) and its role in SAH has been extensively studied (40,59,60,134,135). Indeed, the role of Hp in Hb scavenging, has also been known for many years (136) however the mechanisms and its involvement in several disease processes involving the vascular and neurological systems, are more recent discoveries (71,76–78,137–140).

Galea et al showed that there is profound relative and absolute hypohaptoglobinorrhachia in SAH patients, demonstrating that the Hb/Hp/CD163 scavenging system is saturated and unable to cope with the Hb burden produced by the SAH (73). Their concept of a calculated "total Hb binding capacity of Hp" was used to demonstrate this, showing that in humans at least, there is not enough Hp to deal with the Hb burden. However, this does not appear to be the case in the rat. Whilst the average level of Hp in clinical SAH was in the order of 799ng/ml, the rat at the peak period of free Hb at 24hrs was able to produce an average of over 12,000ng/ml. This suggests that in the rat there is a robust Hb clearing response and that Hp

more than meets the challenge posed by the free Hb liberated in SAH. Our study goes ones step further than Galea et al (73), by creating a temporal profile of the release of Hb from the subarachnoid clot, and analysis of the Hp and sCD163 levels over the same period. What we have demonstrated is that there is evidence of free, purportedly toxic, extra-cellular Hb almost from the immediate onset of SAH. This has implications for the interpretation of future studies into SAH attempting to grapple with competing theories of early brain injury (42,43,141)and others involving soluble factors contributing to the delayed brain injury seen in SAH (60).

Perhaps the most important finding is that not only is there an increase in Hp following SAH in the rat, but there appears to be a direct relationship between the Hb/Hp/CD163 scavenging system and the clearance of free Hb. Specifically – there is an acute, appropriate surge in the major Hb scavenging molecules in the CSF following the entry of pathological quantities of free-Hb into the CNS. This results in the rapid clearance of Hb, as would be expected in the systemic circulation. This is in direct contrast to what is understood to happen in SAH in humans.

Several questions arise in light of this. Zhao et al have shown that Hp can be synthesised within the CNS by oligodendrocytes in a model of intracerebral haemorrhage (142). However, Yang et al demonstrated that increased Hp following traumatic brain injury is mainly synthesised in the liver, and that its presence in the CNS may be attributable to dysfunction of the blood brain barrier (131). Which poses the question, what is the case of alterations in Hp levels following SAH? This and other questions do arise from our investigation, namely, if the Hb/Hp/CD163 pathway is so efficient in the rodent, why is this not the case in humans? Clearly, further studies are required to address these questions. However, it does highlight the

fact that the effects of inter-species differences in pre-clinical studies cannot be discounted. Furthermore, such discrepancies between experimental models and clinical findings really emphasises that verification of any experimental findings should be replicated in multiple models using multiple species before application to the clinical setting (143). In the current study, the SAH model and species chosen may not accurately reproduce key aspects of the clinical condition and this may, at least in part, account for the discrepancies observed.

The significant rise in Hp levels at 24hrs and 48hrs post-SAH, coupled with evidence of increased sCD163 and CD163 staining is telling, suggesting that the mechanisms for Hb removal in the systemic circulation also exist within the CNS. The results show that the free Hb was effectively cleared by 72hrs post-SAH, suggesting that perhaps the clearance mechanisms in the rat are adequate to deal with the amount of Hb produced in this model of SAH at least, perhaps with the help of auxiliary, Hp-independent, Hb scavenging pathways (74). However, there is still a period that free Hb, whose toxic effects are not effectively neutralized by being completely complexed by Hp molecules (57,69,70,76,137,138), is present within the CNS to contribute to the pathological processes underlying cerebral vasospasm and delayed brain injury mechanisms following SAH.

The significance of the immunohistochemical results is unclear. These studies were performed in an attempt to provide evidence for upregulation CD163 within the brain substance, which may further clarify the pathways that lead to the eventual removal of Hb from the CNS. Though there appears to be a profound soluble response within the CSF, this is not appreciably replicated within the parenchyma of the brain itself. This perhaps suggests that the main site of Hb scavenging is in fact in CSF.

3.5 Summary

In a rat model of SAH we have demonstrated that toxic extracellular Hb is released almost immediately following the initiation of bleeding. By 24hrs following SAH, the levels of Hp and the Hb/Hp scavenging receptor are increased significantly from baseline, with effective clearance of Hb from the CSF occurring within72hrs, highlighting the importance of the role that Hp plays in SAH. This study sheds further light on the way Hb is handled in the CNS after SAH, and may point to future therapies for DND in these patients. It also suggests that there is a profound difference in the way that Hb is dealt with in rats compared to humans, which has implications for their use as an experimental model in SAH and delayed cerebral ischaemia research. Future studies are required to investigate the therapeutic potential of exogenous Hp in treating SAH both in large animal models and in clinical SAH.

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Conflicts of interest

Disclosures: None

Chapter 4
General Discussion

This is the first study to describe a temporal profile demonstrating the relationship between Hb, Hp and sCD163 levels in SAH in any species, and the first to clearly show that Hp plays a significant role in the clearance of free Hb in the setting of SAH. What these results demonstrate is a putative relationship between the Hp-CD163 scavenging system and Hb in SAH. A clear increase in Hp and sCD163 levels, in response to elevated levels of free Hb, concurrent with clearance of Hb from the CSF, suggest that this mechanism plays a significant role in Hb clearance in the CNS.

4.1 Subarachnoid Haemorrhage & Delayed Cerebral Ischaemia

Antiquated definitions of the syndrome of neurological deterioration following SAH have been challenged recently (144). Whilst the complex nature of this syndrome has been appreciated for decades (145), the true pathophysiology underlying it is still poorly understood. Originally thought of in simple mechanistic terms, study into the aftermath of SAH now encompasses broad fields ranging from vascular pathobiology to neuroimmunology (117,146–149). Whilst it is now clear that there is a definite dissociation between angiographic cerebral vasospasm and actual clinical outcomes after SAH (28,46,50), this is not to say that the same mechanism is not responsible, at least in part, for these two processes occurring simultaneously. Indeed, cerebral vasospasm and delayed cerebral ischaemia are almost certainly intimately linked, though a clear explanation of this relationship is lacking.

With this consideration in mind, research methods for investigating SAH and DCI have similarly evolved (84,92). There has been less of a focus on the pathophysiology of arteriopathy following exposure to extravasated blood, to more on the delayed mechanisms that occur as cascade events following the initial insult, including qualitative analysis of CSF components following SAH such as this study (41,42,44,45,122,124,134,150–153). Despite efforts to counter the delayed effects of SAH, and critically, the search for therapies that lead to improvements in outcomes for patients suffering this condition, have essentially been fruitless to date (37,38,126). While the benefits of the calcium channel inhibitor nimodipine have been confirmed in large multicentre clinical trials (36), the success of more novel therapies has been limited to pre-clinical experimental data (154–156).

Possibly the most influential body of research to date, which has profoundly affected our understanding of the relationship between cerebral vasospasm and delayed cerebral ischaemia, were studies investigating the effect of clazosentan. Despite criticism of the study designs, trials investigating the efficacy of this drug, an endothelin-1 receptor antagonist, failed to yield any significant improvement in patient outcomes despite a significant improvement the degree of angiographic spasm in study participants (47,48,119). The failure of this drug is best understood when considering the results of Sabri *et al* and Chen *et al*, who demonstrated in rodent models that there is a dissociation between the beneficial effects of clazosentan on cerebral vasospasm and the delayed secondary effects that are now considered fundamental in our understanding of the pathophysiological basis of poor outcomes in patients suffering delayed cerebral ischaemia (46,50).

4.2 Subarachnoid Haemorrhage & Brain injury

Indeed, the complex sequelae of SAH extends beyond the anatomical realm, with evidence of its effects seen in memory systems and cognition and learning (153,157–159). The role of cell signalling and autophagy has dominated the pre-clinical research of late, with new therapies aiming at ameliorating the effects of cellular and molecular alterations suffered as a consequence of both the intracranial haemodynamic effects of SAH, and the markedly altered constitution of the cerebral microenvironment (42,122–124,160,161). This study adds one more piece to the puzzle, by elucidating the important role that the Hp/CD163 scavenging system plays in this microenvironment.

4.3 Haemoglobin

The recent focus on the molecular mechanisms that play a role in the pathophysiology underlying the poor outcomes for patients following SAH hasn't diminished the purported significance of soluble factors in the pathogenesis of delayed cerebral ischaemia. While it remains one of the more rudimental concepts in SAH research, the theories and research into this area to date have been quite robust (24,40,59,60,134,135,162–168). Free haem acts as a potent cytotoxic pro-oxidant, owing to the Fe atom contained within its protoporphyrin ring, and has been implicated in immune-mediated inflammatory diseases and can sensitize non-haematopoietic cells to undergo programmed cell death in response to a variety of pro-inflammatory agonists (164).

Several studies have demonstrated that extra-cellular Hb causes angiographic cerebral spasm, concurrent with the period of clinical deterioration in patients suffering SAH (40,59). Dreier et all showed that Hb caused spreading ischaemia and focal necrosis in the cerebral cortex of rats (60), and Lara et al demonstrated that exposure of cortical neurons to Hb and haem resulted in an oxidative stress condition, and that neurons were more susceptible to Hb toxicity than astrocytes (169). In a clinical study Suzuki et al showed that patients who had higher levels of Hb breakdown products (and by inference, higher levels of haem oxygenase), had significantly less incidence of symptomatic vasospasm (165), suggesting that Hb clearance mechanisms play a vital role in the development of delayed cerebral ischaemia.

The rapid appearance of extra-cellular Hb in this study, reaching significant levels almost immediately, suggest that Hb toxicity potentially begins extremely early following SAH. This fact should be considered in light of the competing theories surrounding delayed cerebral ischaemia – delayed brain injury versus soluble "spasmogenic" factors. Our findings suggest that the inciting mechanism in both theories (i.e. catastrophic intracranial haemodynamic changes setting off a cascade of cellular apoptotic mechanisms versus the liberation of a soluble toxic factor into the central nervous system) is present essentially from the moment of ictus, which has major implications for the interpretation of results into this condition, at least in models using the rat.

4.4 Haptoglobin & CD163

Whilst the total volume of extravasated blood in a rat SAH is an order of magnitude less than that sustained in the human condition, the relative swift clearance of Hb in rat SAH does highlight possibly just how significant the difference in Hp levels between the two species is.

Whilst humans appear to mount a rather meagre Hp response, profoundly saturated by free Hb, the rat by comparison produces Hp levels that are more than able to deal with the Hb burden. Hp binds Hb at a ratio of 1.4:1 (weight for weight), thus the measured levels of Hp (over 1mg/ml) at the peak of free Hb at 24hrs (~0.3mg/ml) more than manages to cope with the Hb burden. The temporal profiles indicate a very close relationship between the involved analytes, and this relationship is explainable physiologically. Several questions arise from these results, the most obvious of which is why are the Hb scavenging mechanisms in humans so poor when they are so efficient in the rodent?

The elevation in sCD163, concurrent with the dramatic rise in Hp levels appears to confirm that the Hb is in fact being complexed with Hp and taken up by resident macrophages, rather than this simply being a casual relationship. Additionally, taking into account the haptoglobinorrhachia seen in human SAH, the sCD163 response suggests that the rate-limiting component of the system is the Hp. Our understanding of Hp and its role in neurological disease is evolving, rather than this protein being simply a scavenging molecule, it is clear that it has an important anti-inflammatory role.

The removal of free Hb by Hp occurs in three distinct biochemical events – Hb-Hp complex formation (one of the strongest non-covalent binding relationships seen in plasma), oxidative inactivation, followed by clearance. Hb-Hp binding is of high affinity (one of the highest reported in plasma), with a slow dissociation rate, and occurs in a ratio of 1:1 stoichiometrically (1.4:1, weight for weight). In humans Hp1-1 has the highest affinity for Hb dimers, and the differing variants have slight differences in their biochemical activity and their other ancillary roles. Hp polymorphism has been association with many inflammatory diseases, explained by

a phenotype-dependent modulation of oxidative stress and prostaglandin synthesis (170). More recently, recognition that Hb-Hp clearance leads to the production of anti-inflammatory metabolites (carbon monoxide, bilirubin, ferritin (57)) through the HO-1 pathway and various signalling cascades triggered by Hb-Hp binding to CD163 has shed further light on its involvement in disease processes (70).

Zhao et al showed that Hp has a neuro-protective role in intracerebral haemorrhage, and that oligodendroglial derived Hp plays a protective role against Hb-mediated toxicity to neurons and oligodendrocytes (142). Multiple authors to date have emphasised the importance of Hp's role in SAH, however the majority have focussed on the differences that the phenotypical subtypes exert on the manifestation of cerebral vasospasm (generically) (65–67,128).

4.5 Implications and Future Directions

Given that Hb has demonstrated toxicity in the CNS, and in this study it is effectively cleared from the CSF in the presence of adequate levels of Hp, suggests that Hp augmentation has a potential therapeutic role in SAH and delayed cerebral ischaemia in humans, where a state of relative and absolute hypohaptoglobinorrhachia is seen in SAH. It is significant that the rodent model of SAH does not replicate the Hb/Hp discrepancy that is present in human SAH, suggesting that alternate models are required to investigate this further and determine whether supplementary treatment is successful. Dose-response and therapeutic window studies for Hp would be required to determine which dose is most effective, and how long the window for treatment is.

This study also has implications for the future use of rodent models of SAH, given how profoundly different the Hb scavenging mechanisms are between the two species. Indeed, this model is possibly more representative of what might actually happen if humans who suffered a SAH were treated with Hp. While this study does call into question the validity rat models of SAH, its future use may in fact provide insights into the possible Hb clearance pathways, which may be taken advantage of in humans, using haptoglobin as a therapeutic agent.

Future studies examining the Hb/Hp/CD163 scavenging pathway in SAH may consider using different species, such as the sheep, to see if this phenomenon is consistent in non-human species.

4.7 Study Limitations

This study has several limitations:

- The major criticisms of transitional models for neuroscience (particularly stroke) research range from criticism of the choice of model (with genetic differences, different hypoxia tolerance and neural regenerative capacity between species), to the use of anaesthesia (which can have neuroprotective properties), and the fact that many positive findings in pre-clinical studies are not borne out in clinical trials (92,171–173). In any case this study actually seems to gives further weight to these criticisms.

- This study does not explain why there is such an effective Hb clearance mechanisms in the rat, apart from showing that it exists. Where is the Hp produced, systemically or locally? If it is produced outside the central nervous system, how does it cross the BBB in rats

so readily when this appears to be a major impediment to the influx of systemic Hp into the CNS of humans?

- Additionally, although the averaged data shows that Hp levels not only meet but also exceed the levels required to scavenge the available Hb, it was not calculated in a pairwise fashion. Nonetheless the data was significant and diametrically different to the overall situation seen in humans.

- Whilst it was clearly demonstrated that the free Hb was removed from the CSF, it's precise fate was not elucidated. Though the implication is that it was scavenged by the abundant Hp and endocytosed by phagocytes, this is purely supposition and was not demonstrated directly.

4.8 Conclusions

This study establishes a temporal profile of Hb, Hp and sCD163 in a rat model of SAH. The profile reveals that the rat is able to rapidly respond to a pathological burden of free-Hb in the CNS, with rapid appearance of Hp at levels more than capable of scavenging the available Hb. This is in direct contrast to the situation seen in humans, where a relative and absolute hypohaptoglobinorrhachia prevails.

This study has major implications for the use of and interpretation of results of rodent model SAH studies. It also supports the theory that haptoglobin augmentation in human SAH may have therapeutic merit.

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