Brief Report

The heme-hemopexin scavenging system is active in the brain, and associates with outcome after subarachnoid hemorrhage

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Abstract

Background and Purpose – Long-term outcome after subarachnoid hemorrhage (SAH) is potentially linked to cytotoxic heme. Free heme is bound by hemopexin (Hpx) and rapidly scavenged by CD91. We hypothesized that heme scavenging in the brain would be associated with outcome after haemorrhage.

Methods - Using cerebrospinal fluid (CSF) and tissue from SAH patients and control individuals, the activity of the intracranial CD91-Hpx system was examined using enzyme-linked immunoassays, ultra-high performance liquid chromatography and immunohistochemistry.

Results - In control individuals, CSF Hpx was mainly synthesized intrathecally. After SAH, CSF Hpx was high in one-third of cases, and these patients had a higher probability of delayed cerebral ischaemia and poorer neurological outcome. The intracranial CD91-Hpx system was active after SAH since CD91 positively correlated with iron deposition in brain tissue. Heme-Hpx uptake saturated rapidly after SAH, since bound heme accumulated early in the CSF. When the blood-brain barrier was compromised following SAH, serum Hpx level was lower, suggesting heme transfer to the circulation for peripheral CD91 scavenging.

Conclusions - The CD91-heme-Hpx scavenging system is important after SAH and merits further study as a potential prognostic marker and therapeutic target.

Introduction

SAH is an example of extravascular haemolysis with a high mortality and morbidity, and associated economic cost. Following haemolysis, cell-free Hb undergoes oxidation to methemoglobin, which through a number of hemichrome intermediates, finally degrades into a denatured globin protein and the redox-active heme moiety (iron-protoporphyrin IX). Due to a combination of redox activity and lipophilicity, free heme is toxic in a number of ways; including, covalent modification of substrates, intercalation in the lipid bilayer and lipid peroxidation, which perturbs membrane homeostasis to cause cellular dysfunction and cell death.[1] Hpx neutralizes the redox toxicity of heme by formation of the heme-Hpx complex, which prevents heme from generating free radical reactions[2], and leads to its uptake by CD91.[3] The CD163-haptoglobin system is the body's first line of defence during haemolysis, but this system is saturated after SAH, and free Hb is detectable in the CSF.[4] In this situation, the CD91-Hpx system is likely to be important.

Hpx is an abundant plasma protein, also expressed by neurones and glia.[5] The relative contribution of these two sources to human CSF Hpx is unknown. Also, the response of the human Hpx-CD91 scavenging system to subarachnoid hemorrhage has not been studied. This study addresses these questions by analysing CSF and brain tissue from patients after SAH and controls.

Materials and Methods

Clinical Studies. Participants were recruited after referral to tertiary centres in Manchester, Birmingham, Southampton and Cambridge, with respective Research Ethical Committee

approvals. The characteristics of control participants (n=20) and SAH patients (n=30) in the main study are listed in Table I (online-only Data Supplement). CSF in patients with SAH was obtained from external ventricular drains. Control participants were patients with non-inflammatory / non-haemorrhagic conditions who underwent lumbar puncture and were subsequently found to have normal CSF with respect to protein, glucose, cell count, cytology, albumin CSF/serum quotient and isoelectric focusing for oligoclonal bands. Seven SAH patients were recruited to Clinical Study 2, to enable further analysis of heme, which required more CSF (Table II, online-only Data Supplement). Heme quantitation was performed using an in-house validated ultra-high performance liquid chromatography (UPLC) technique; established immunoassays were used for Hpx and albumin (online-only Data Supplement).

Post-mortem study. *Post-mortem* brain tissue from SAH (n=7) and matched control (n=5) cases was obtained from the University Hospital Southampton NHS Foundation Trust as part of the UK Brain Archive Information Network (BRAIN UK). Tissue sections were selected close to the bleed, so anatomical region varied in individual cases. The mean SAH-to-death interval was 16 days (range 11-25 days); further characteristics are available in the online-only Data Supplement. CD91 and iron were analysed by immunohistochemistry and Perls staining respectively. See online-only Data Supplement for more details.

Statistical analysis. The distribution of each dataset was assessed, and parametric or non-parametric tests were employed accordingly, as indicated in the text. Statistical tests were conducted at the 5% two-sided significance level using SPSS v21.

Results

Hemopexin is mainly produced intrathecally in control CSF. The CSF Hpx reference range as determined by enzyme-linked immunosorbent assay was 12.3 to 32.6µg/ml; the mean concentration was 22.4µg/ml. There was no gender difference (means of 21.5µg/ml and 24.6µg/ml in females and males respectively, p=0.23, unpaired t-test). A well-established and accepted technique to determine intrathecal synthesis of blood-derived proteins similar to albumin is the intrathecal index, defined as the CSF/serum ratio of Hpx (Q_{Hpx}) divided by the CSF/serum ratio of albumin (Q_{alb}).[6] Albumin is a plasma protein which is not synthesized in brain and is wholly derived from plasma via diffusion across the blood-brain barrier (BBB). With a molecular weight of 69 kDa and a molecular size of 25.6 Å, albumin has the appropriate biophysical parameters to act as a reference protein for the diffusion of Hpx across the BBB (Hpx: molecular weight of 68 kDa; molecular size of 36 Å). Q_{Hpx} was significantly greater than Q_{alb} ($p<0.0001$, Wilcoxon test, Figure 1A); the intrathecal index (Q_{Hpx}/Q_{alb}) was 10.5. Thus the vast majority of Hpx is produced intrathecally in control individuals, with only about one tenth being derived from the circulation.

The CD91-hemopexin heme scavenging system is present and active in human brain after SAH. CD91 immunohistochemistry on human brain tissue revealed expression in neurons and glia (Figure 1B). Uptake of heme-Hpx complexes leads to intracellular deposition of heme's iron moiety.[7] Perls staining to quantify iron deposition revealed a significantly greater deposition of iron in SAH *versus* control cases (p=0.028, Mann-Whitney test, Figure 1C). Regardless of whether there was blood clot in the sections, iron deposition significantly correlated with CD91 (Spearman r=0.79, p=0.0025, Figure 1D), indicating that the CD91-Hpx system actively scavenges heme after SAH.

Increased CSF hemopexin after SAH is associated with poor outcome. After SAH, CSF Hpx had a bimodal distribution; in 30% of patients (n=9/30), CSF Hpx was above the reference range (Figure 2A). In high *versus* normal CSF Hpx patients, delayed cerebral ischaemia (DCI) occurred more frequently and neurological outcome six months after SAH, as assessed by the modified Rankin Scale (mRS), was poorer (DCI: 57% *versus* 11%, p=0.028, Fisher exact test; mean mRS: 5.0 *versus* 2.4, p=0.025, unpaired t-test). The difference in Glasgow Outcome Scale (GOS) was not statistically significant (mean 2.5 *versus* 3.9, p=0.122, unpaired t-test). The difference in outcome between high and normal CSF Hpx groups could not be explained by a number of other factors related to bleed size or severity (Table III, online-only Data Supplement). Although more females than males had a high CSF Hpx, this was not significant, and CSF Hpx was not significantly different between the sexes (25.2µg/ml and 23.3µg/ml in females and males respectively, p=0.699, Mann-Whitney test). Overall, this indicates that CSF Hpx level is associated with outcome after SAH, and may be a potentially useful prognostic marker, independent of bleed size.

Source of increased CSF hemopexin after SAH. The seven-fold increase in CSF Hpx (Table III, online-only Data Supplement), could be blood or brain-derived. Q_{Hpx} was significantly raised in high CSF Hpx patients (three-fold, p<0.001, unpaired t-test), indicating partial intracranial origin (Figure 2B). The blood-derived fraction could have two sources: the initial bleed itself, and increased transfer from the circulation via a more permeable BBB. If the initial bleed was the predominant source of plasma proteins in the CSF, one would expect a significant negative correlation between sampling time and Q_{alb}; however this was not present (Pearson correlation coefficient = -0.045, p=0.826). In support of increased transfer from the circulation across a compromised BBB, there was a significant increase in BBB permeability in the high CSF Hpx group as evidenced by a raised Q_{alb} (two-fold, p=0.033,

unpaired t-test, Figure 2C). Hence, the predominant source of increased CSF Hpx associated with poor outcome was intrathecal, with some derived from the circulation.

Saturation of heme-Hpx uptake following SAH. The high CSF Hpx suggested saturation of heme-Hpx uptake. In order to look for this, SAH CSF was examined for the presence of heme-Hpx complexes. Sufficient CSF from one patient in Clinical Study 1 was available for UPLC analysis to detect bound heme; in this patient, who had a high CSF Hpx level (98.5µg/L), a substantial amount of heme was bound to Hpx and albumin (Inset in Figure 3A, Figure I online-only Data Supplement, peak at 4.5min). In seven other high-grade SAH patients, with repeated CSF sampling (Clinical Study 2), bound heme was found at all time points, confirming rapid saturation of heme-Hpx uptake, up to at least day 13 after SAH (Figure 3A).

Free heme after SAH. In the same seven patients from Clinical Study 2, unbound heme was assayed by performing UPLC before and after adding recombinant Hpx to the CSF sample, the difference in area-under-the-curve representing unbound heme. All samples tested showed an increase in the peak for bound heme when pre-incubated with recombinant Hpx, indicating the presence of free, unbound heme in the CSF, up to at least day 13 after SAH (Figure 3B).

Evidence for efflux of free heme from the brain. After SAH, a small and lipophilic molecule such as heme can theoretically diffuse out of the brain into the bloodstream down a steep concentration gradient across the BBB. There was a significant drop in serum Hpx after SAH (mean of 0.72mg/ml in controls *versus* 0.55mg/ml after SAH, p=0.002, unpaired t-test,

Figure 3C). This decrease was confined to patients with the highest Q_{alb}, in whom BBB permeability to heme would be the highest (p=0.04, unpaired t-test, Figure 3D).

Discussion

This is the first study to characterise the intrathecal CD91-Hpx system in control individuals and following SAH. Intrathecal production is likely to be the main source of CSF Hpx, with only about one tenth being derived from the circulation. Hpx is known to be produced within the brain and CD91 is not expressed by cerebral endothelium[5, 8], so receptor-mediated transport of Hpx into the brain is unlikely, but cannot be excluded. Still, CSF Hpx was 10-fold lower compared to serum, suggesting that the brain has a comparatively lower heme-binding capacity.

After SAH, the CD91-Hpx scavenging system is active in the brain, since CD91 significantly correlated with iron deposition. CSF Hpx had a bimodal pattern after SAH. Despite similar bleed size and severity, high CSF Hpx patients had a poorer outcome, with higher DCI rates and higher mRS scores. This needs replication since CSF Hpx may be of clinical utility as a prognostic marker. The GOS was not significantly different between individuals with normal and high CSF Hpx; however, such a discrepancy between GOS and mRS has been noted after SAH before, [9] and may be related to the higher number of scoring categories of the mRS being able to differentiate neurological sequelae with greater subtlety. In addition mRS is a stroke outcome scale, more akin to SAH, while GOS was developed as a scale for use in traumatic head injury.

The elevation of CSF Hpx, and its relationship to outcome, is intriguing and the mechanism is not clear. Hpx has been reported to be neuroprotective[10], and therefore CSF Hpx is not assumed to be toxic. However it is also possible that when CSF Hpx is too high, it becomes deleterious by binding heme, preventing its efflux from the brain and resulting in intracellular heme/iron overload, which may be toxic to neurones/glia. Another potential explanation is that events after SAH, such as saturation of heme-Hpx uptake and/or inflammation, result in high CSF Hpx and poor outcome, which are therefore indirectly associated. Clearly further mechanistic studies are needed.

CSF Hpx had two sources: (1) accumulation from an intrathecal origin (as indicated by a three-fold rise in the Hpx intrathecal index); (2) increased transfer from the circulation (supported by a two-fold higher Q_{alb}). Accumulation of brain-derived Hpx after SAH may occur as a result of increased synthesis or decreased CD91-mediated scavenging. Increased synthesis may be secondary to the host inflammatory response, since the Hpx promoter is IL-6 responsive[11] and IL-6 levels are elevated in SAH CSF.[12] Decreased CD91-mediated scavenging may be due to plateauing in heme-Hpx uptake since heme and Hpx were detected simultaneously in the CSF. Also, since CD91 has multiple ligands, it is possible that there is competition for CD91 from other ligands, such as ApoE[13]. Interestingly, CSF ApoE is known to decrease following SAH[13] and low CSF ApoE associates with a poor outcome,[14] similar to patients with high CSF Hpx in this study.

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Figures

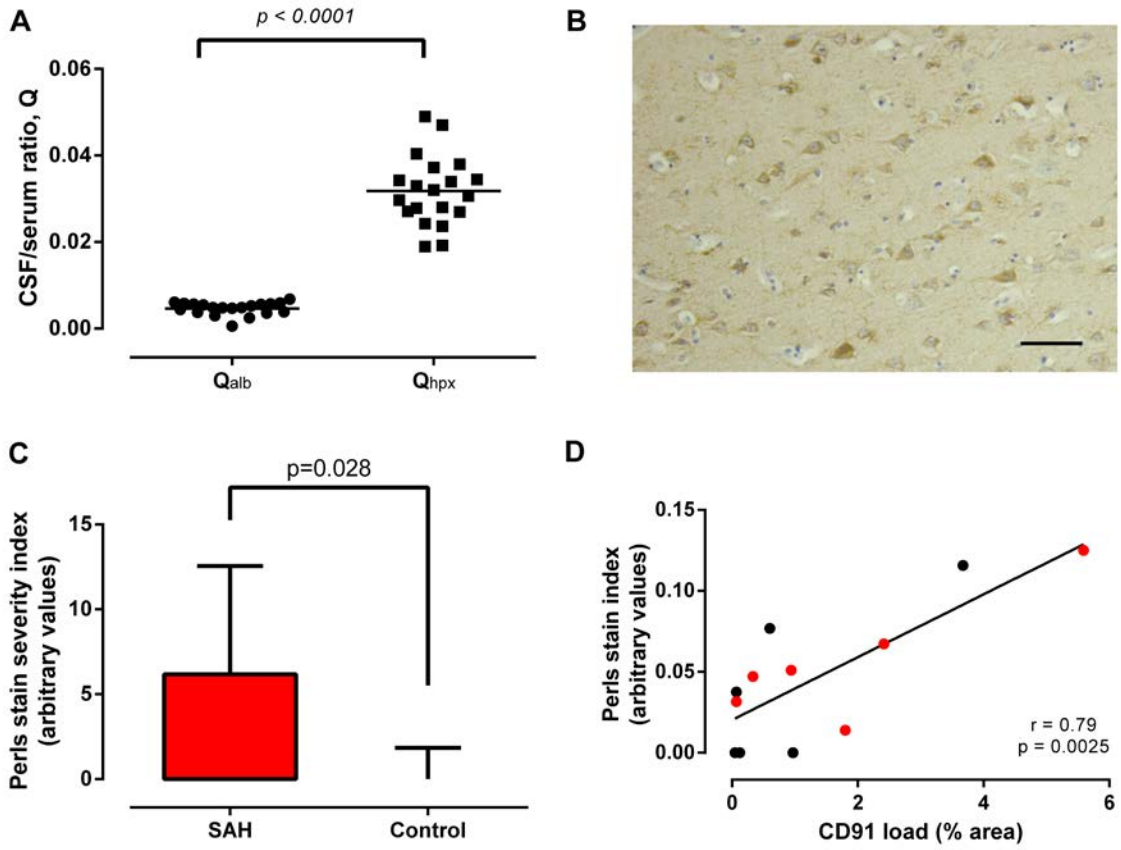


Figure 1 A, Albumin (Q_{alb}) and hemopexin (Q_{hpx}) quotients in controls; **B-D,** Post-mortem study; **B,** CD91 immunohistochemistry in control human brain; scale bar: 100µm; **C,** Perls staining for iron deposition in SAH and control cases; **D,** Correlation of CD91 with intracellular iron after SAH; red and black data points denote SAH tissue sections with and without blood clot respectively.

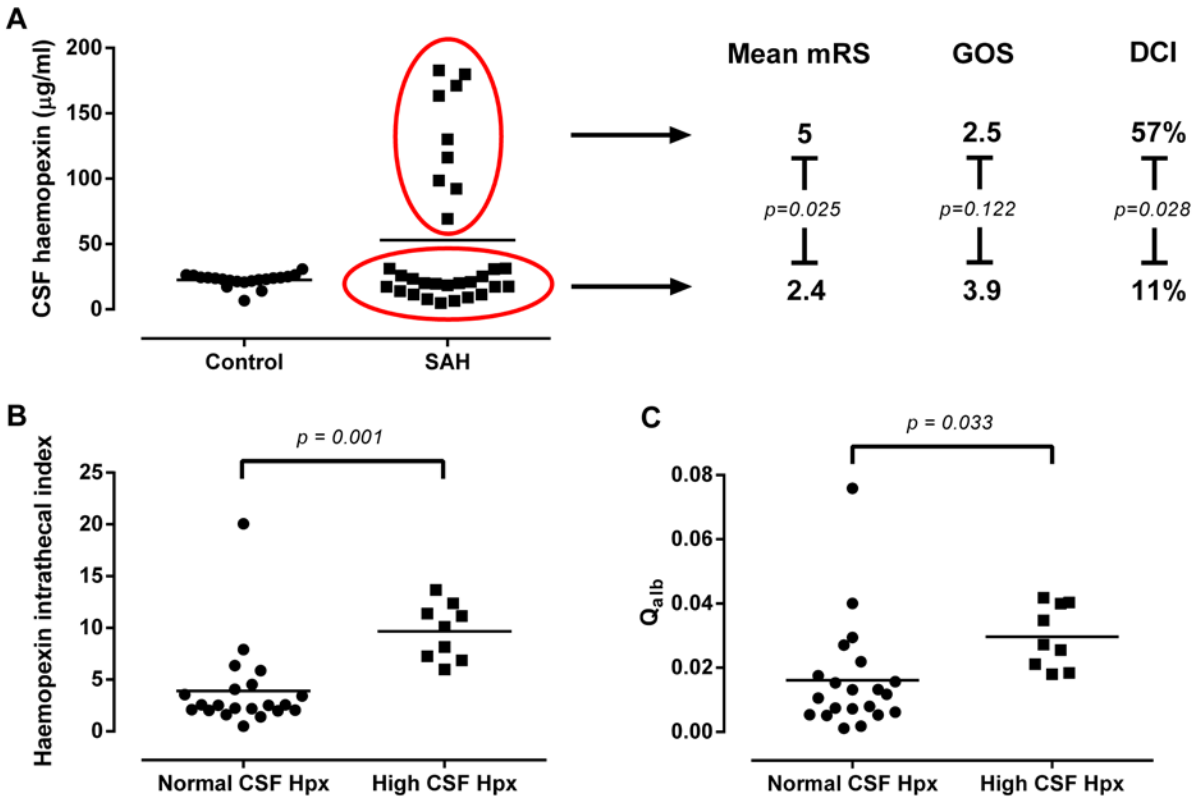


Figure 2 A-C, CSF Hpx from controls and SAH patients; **A** DCI, GOS and mRS in high *versus* normal CSF Hpx SAH patients; **B-C,** Hpx intrathecal index and albumin quotient of high *versus* normal CSF Hpx SAH patients.

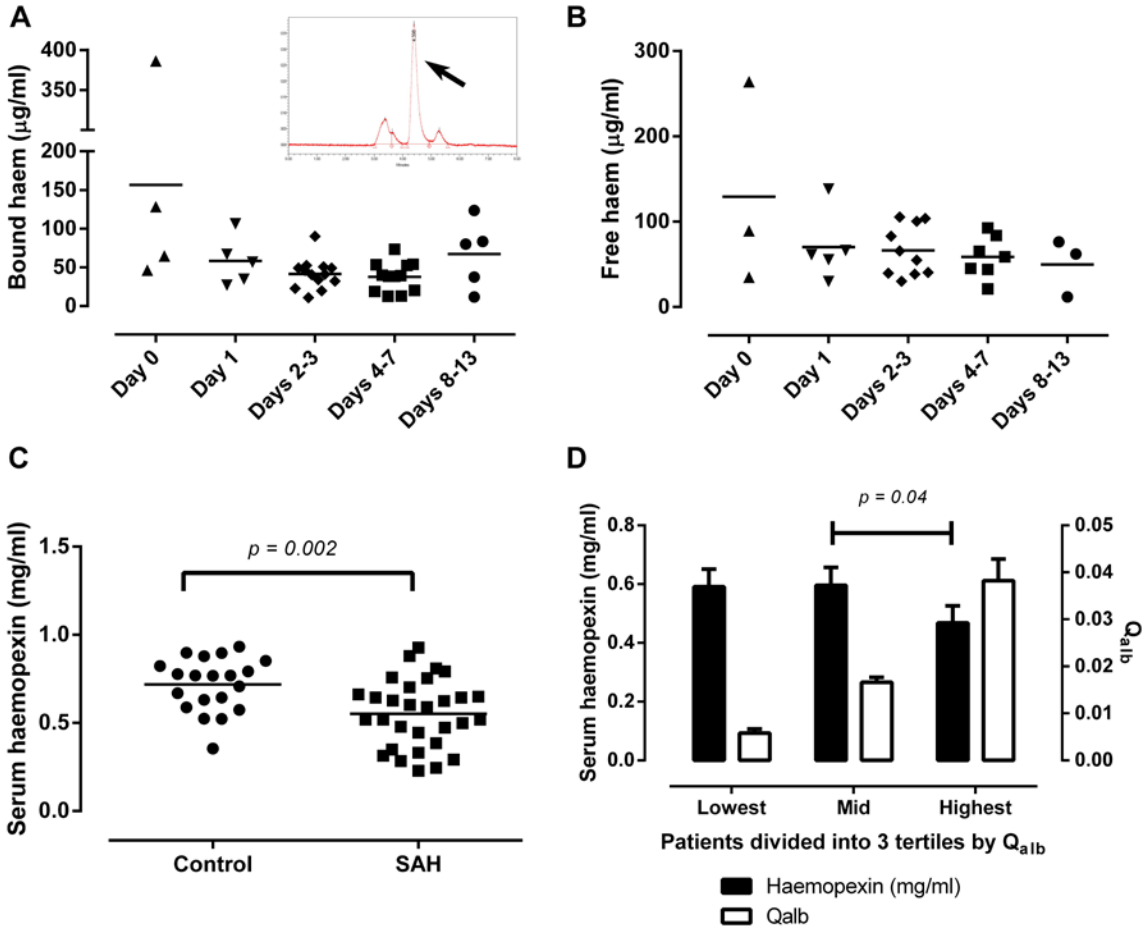


Figure 3 A-B, Bound and free heme at day 0 up to 8-13 days post-ictus. Inset shows UPLC analysis of a high Hpx CSF: a substantial peak for Hpx/albumin-bound heme is observed at 4.5min (arrow); **C-D,** Heme efflux to the periphery after SAH. **C,** Serum Hpx in controls and SAH patients. **D,** Variation of serum Hpx level with albumin quotient.

SUPPLEMENTAL MATERIAL

The heme-hemopexin scavenging system is active in the brain, and associates with outcome after subarachnoid hemorrhage

Supplemental tables

Table I Demographics of Clinical Study 1

	SAH	**Controls**
Number	30	20
Age (years)	52.1 ± 9.6	41.3 ± 16.6
Sex (% female)	68	70
Presentation Glasgow Coma Score[a]	11 ± 4.2	
Fisher grade[a]	3.7 ± 0.7	
WFNS score[a]	2.9 ± 1.5	
DCI: clinical evidence (%)[a]	23.1	
DCI: CT evidence (%)[a]	23.1	
DCI: clinical & CT evidence (%)[a]	30.7	
6 month Glasgow Outcome Score[b]	3.5 ± 1.7	
6 month Modified Rankin Score[c]	3.0 ± 2.5	
Days post-ictus when sample taken	3.9 ± 2.6	

Values represent mean ± standard deviation where applicable. Data available for 26[a], 28[b] and 25[c] patients. Abbreviations: CT: Computed Tomography; DCI: Delayed Cerebral Ischaemia; WFNS: World Federation of Neurological Surgeons

Table II Demographics of clinical study 2

Number	7
Age in years	59.9 ± 17.8
Sex (% female)	40
Presentation Glasgow Coma Score	9.7 ± 3.7
Fisher grade	3.9 ± 0.4
WFNS score	3.0 ± 1.3
DCI: clinical evidence	2/7
DCI: CT evidence	1/7
DCI: clinical & CT evidence	2/7
3 month Modified Rankin Score	2.2 ± 1.5

Values represent mean ± standard deviation where applicable. Abbreviations: CT: Computed Tomography; DCI: Delayed Cerebral Ischaemia; WFNS: World Federation of Neurological Surgeons

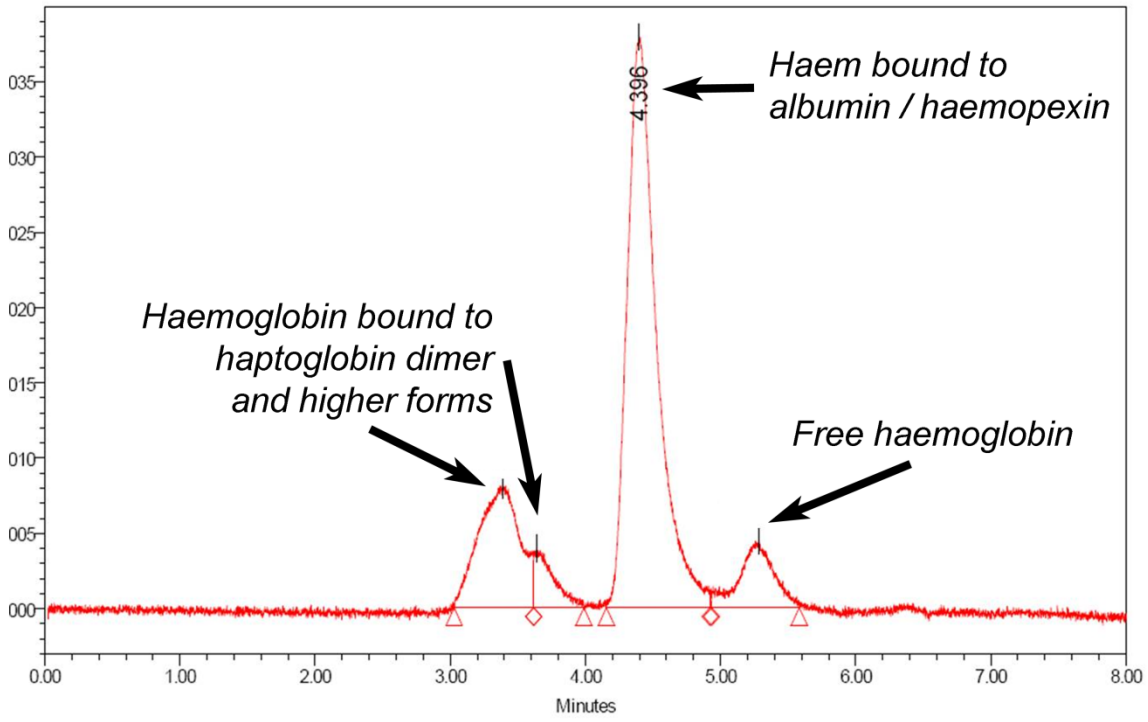
Table III Characteristics of patients by CSF Hpx concentration

	Normal CSF Hpx	**High CSF Hpx**	***p* value**
Number of cases	21	9	
CSF Hpx concentration (µg/ml)	18.3 ± 8.0	133.8 ± 42.2	1.4×10^{-7}*
DCI: clinical evidence (%)[a]	11	57	0.028*
DCI: CT evidence (%)[a]	11	57	0.028*
DCI: clinical & CT evidence (%)[a]	21	57	0.101
6 month Modified Rankin Score[b]	2.4 ± 2.2	5.0 ± 2.4	0.025*
6 month Glasgow Outcome Score[c]	3.9 ± 1.5	2.5 ± 1.6	0.122
Presentation GCS	11.7 ± 3.6	9.0 ± 5.4	0.248
Fisher grade	3.6 ± 0.8	3.9 ± 0.4	0.497
WFNS score	2.7 ± 1.5	3.3 ± 1.6	0.395
Days post-ictus sample taken	4.4 ± 2.6	2.6 ± 2.2	0.120
CSF Hb concentration (µmol/l)[d]	0.69 ± 0.93	0.20 ± 0.18	0.219
Blood clot: mean voxel radiodensity[e]	103.1 ± 11.1	110.2 ± 6.3	0.198
Age in years	51.1 ± 9.0	54.4 ± 10.0	0.388
Sex (% female)	55	89	0.207

Values represent mean ± standard deviation where applicable. Data available for 26[a], 25[b], 28[c], 23[d] and 20[e] patients. Statistical analysis employed Mann-Whitney test (for CSF Hpx, Fisher, WFNS), Fisher exact test (for DCI and gender) and unpaired test for the rest. Abbreviations: CSF: Cerebrospinal Fluid; CT: Computed Tomography; DCI: Delayed Cerebral Ischaemia; Hb: Haemoglobin; Hpx: Haemopexin; WFNS: World Federation of Neurological Surgeons

Supplemental figures

Figure I The CD91 system is saturated following SAH. UPLC trace at 415nm to identify bound heme related peaks from the CSF of a patient with high CSF Hpx; a substantial peak for Hpx and albumin bound heme is observed at 4.5min



Supplemental Methods

Clinical study 1 (Main study)

Admission computed tomography (CT) images were available for 20 patients, and quantitative analysis of blood clot average voxel radiodensity, as a surrogate of bleed size, was performed as previously described.[1] Clinical information regarding delayed cerebral ischaemia (DCI) was available for 26 patients. Clinical DCI was defined as the onset of a new focal neurological deficit or a two point drop in the Glasgow Coma Score in the absence of rebleeding, hydrocephalus, metabolic abnormalities or seizure activity.[2] Computed tomography (CT) evidence of DCI consisted of low attenuation on unenhanced CT of the brain consistent with ischaemia, irrespective of clinical state, which was not deemed to be a result of surgical intervention. CSF in patients with SAH was obtained from external ventricular drains. Control participants were patients with non-inflammatory / non-haemorrhagic conditions who underwent lumbar puncture and were subsequently found to have normal CSF with respect to protein, glucose, cell count, cytology, albumin CSF/serum quotient and isoelectric focusing for oligoclonal bands. Samples were collected with Research Ethical Committee approval (04/Q2707/236 and 07/H0304/71). Serum and CSF Hpx levels were analysed by enzyme-linked immunosorbent assay (ELISA) using commercially available kits, as per kit instructions (AssayMax Human Hemopexin, EH1001-1 and EH2001-1 respectively, AssayPro, MO, USA). The upper and lower limits of the CSF Hpx reference range was calculated by adding and subtracting two standard deviations around the mean. Serum and CSF albumin were analysed by rate nephelometry on a Beckman Coulter IMMAGE immunochemistry system. Sufficient CSF was available from 23 patients with SAH to perform derivative spectrophotometry for quantification of Hb as

previously described, [1] and from one patient to perform heme measurements using ultra-high performance liquid chromatography (UPLC) as described below.

Clinical study 2

Clinical study 1 did not produce large enough CSF volumes for UPLC analysis of heme concentration which needed 400uL. Therefore, in a second study, serial CSF samples were obtained from seven additional patients with high grade SAH (Fisher grade III-IV) admitted to the Southampton centre, requiring prolonged external ventricular drainage, under research approval 12/SC/0666. The characteristics of these patients are shown in Table II in the online-only Data Supplement.

Ultra-high performance liquid chromatography (UPLC). Size-exclusion UPLC was used to separate components by size. Peaks were monitored at 415 nm wavelength to exclude non-heme related peaks while differentiating bound heme from free and bound Hb. Heme quantitation was performed by reading against a standard curve. A Hb standard solution was prepared from commercially-available lyophilized human Hb (Sigma). This was reconstituted to 1 g/L in diluent (9 g/L NaCl, 10 mM EDTA). The concentration of the standard Hb solution was verified independently by spectrophotometric quantitation of derivatized heme at 570 nm using a Hemocue™ (Hemocue, Sweden). A concentration series of 9 data points from 0 to 1 mg/ml was prepared from the standard solution and measured at 415 nm on the UPLC. Accuracy of the standard curve was determined to be 3.3% using a Hb control. CSF samples were saturated with 250 µg/ml Hpx (Sigma) to detect total heme, or measured neat to detect bound heme. 50µl of each sample was loaded onto the UPLC column using a running buffer consisting of 50 mM Tris and 150 mM NaCl, at pH 7.5. Absorbance was measured at 415 nm and the area under the curve calculated and quantitated against the standard curve. Free heme was calculated by subtracting bound heme from total heme. Each

assay run was controlled using an in-house hemoglobin-haptoglobin standard measured at three concentration levels; 200 µg/ml, 10 µg/ml and 1 µg/ml to cover the dynamic range of the assay, with precision of 1.9%, 16.1% and 36.3% respectively.

Post-mortem study

Post-mortem formalin-fixed paraffin-embedded tissue from a different set of SAH (n=7) and control (n=5) cases was obtained from the University Hospital Southampton NHS Foundation Trust as part of the UK Brain Archive Information Network (BRAIN UK) which is funded by the Medical Research Council (Research Ethical Committee approval 14/SC/0098). At the age of death after SAH, patients had a mean age of 57 years (range 42 to 71). 71% were females. The location of the aneurysm varied (including middle cerebral, posterior communicating and basilar arteries). Hence the location of the clot varied; most sections were taken from the neocortex apart from a cerebellar and brainstem case. Control cases were matched for age (mean age of 53 years, range 35 to 82) and sex (80% females), did not die from neurological causes, and were selected carefully to exclude inflammatory, haemorrhagic or neurodegenerative pathology. **Histology & immunohistochemistry.** Sections were cut at 4µm thickness. To detect the presence of iron deposition, Perls Prussian blue staining was performed. Sections were dewaxed, rehydrated, and treated for 10 minutes with freshly prepared Perls reagent (2% potassium ferrocyanide and 2% HCl mixed in a 1:1 ratio) for 30 minutes. Then the slides were counterstained with 0.1% neutral red, dehydrated and mounted in DePeX. For detection of the CD91 Hpx receptor, immunohistochemistry was performed on paraffin tissue. Sections were dewaxed, rehydrated, pressure cooked in citrate buffer to retrieve antigen, incubated with anti-CD91 antibody (ab92544, Abcam, Cambridge, UK) at 1:1000 dilution overnight, followed by incubation with biotin-conjugated secondary antibody. Development was performed using the ABC method and 0.05% 3,3`-diaminobenzidine.

Sections were finally counterstained with haematoxylin, dehydrated and mounted in DePeX. Sections incubated in the absence of the primary antibody were included as negative controls, and all sections were immunolabelled together to ensure comparability of staining.

Quantification. For each case, ten images were digitally acquired from grey matter in a ribbon following the most prominent sulcus in each section, using a camera mounted on a light microscope at magnification x20. The images were analysed with Image J (version 1.47, NIH US) to obtain a protein load defined as the percentage area stained of total area examined (%). Perls staining severity index was quantified as described previously.[3]

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