Heme–Hemopexin Scavenging Is Active in the Brain and Associates With Outcome After Subarachnoid Hemorrhage

Patrick Garland, PhD; Andrew J. Durnford, MRCS; Azubuike I. Okemefuna, PhD; John Dunbar, MBBS; James A.R. Nicoll, MD; James Galea, PhD; Delphine Boche, PhD; Diederik O. Bulters, FRCS(SN); Ian Galea, PhD

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

Methods—Using cerebrospinal fluid and tissue from patients with SAH and control individuals, the activity of the intracranial CD91–hemopexin system was examined using ELISA, ultrahigh performance liquid chromatography, and immunohistochemistry.

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

Conclusions—The CD91–heme–hemopexin scavenging system is important after SAH and merits further study as a potential prognostic marker and therapeutic target. (Stroke. 2016;47:872-876. DOI: 10.1161/STROKEAHA.115.011956.)

Key Words: brain ■ heme ■ hemoglobins ■ hemopexin ■ subarachnoid hemorrhage

Subarachnoid hemorrhage (SAH) is an example of extravascular hemolysis with a high mortality and morbidity and associated economic cost. After hemolysis, cell-free hemoglobin undergoes oxidation to methemoglobin, which through several hemechromes intermediates, finally degrades into a denatured globin protein and the redox-active heme moiety (iron–proto- porphyrin IX). Because of a combination of redox activity and lipophilicity, free heme is toxic in many 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 Hemopexin neutralizes the redox toxicity of heme by formation of the heme–hemopexin 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 defense during hemolysis, but this system is saturated after SAH, and free hemoglobin is detectable in the cerebrospinal fluid (CSF).4 In this situation, the CD91–hemopexin system is likely to be important.

Hemopexin is an abundant plasma protein and it is also expressed by neurons and glia.3 The relative contribution of these 2 sources to human CSF hemopexin is unknown. Also, the response of the human hemopexin–CD91 scavenging system to SAH has not been studied. This study addresses these questions by analyzing CSF and brain tissue from patients after SAH and controls.

Materials and Methods

Clinical Studies

Participants were recruited after referral to tertiary centers in Manchester, Birmingham, Southampton, and Cambridge, with respective Research Ethical Committee approvals. The characteristics of control participants (n=20) and patients with SAH (n=30) in the main study are listed in Table I in the online-only Data Supplement. CSF in patients with SAH was obtained from external ventricular drains. Control participants were patients with noninflammatory/nonhemorrhagic 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 patients with SAH were recruited to clinical study 2, to enable further analysis of heme, which required more CSF (Table II in the online-only Data Supplement). Heme quantitation was performed using an in-house validated ultrahigh performance liquid chromatography technique; established immunoassays were used for hemopexin and albumin (online-only Data Supplement).

**Postmortem Study**

Postmortem brain tissue from SAH (n=7) and matched control (n=5) cases was obtained from the University Hospital Southampton National Health Service Foundation Trust as part of the UK Brain Archive Information Network. Tissue sections were selected close to the bleed; so, anatomic 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 analyzed by immunohistochemistry and Perls staining, respectively (more details are available in the online-only Data Supplement).

**Statistical Analysis**

The distribution of each data set was assessed, and parametric or nonparametric tests were used accordingly, as indicated in the text. Statistical tests were conducted at the 5% 2-sided significance level using SPSS version 21.

**Results**

**Hemopexin Is Mainly Produced Intrathecally in Control CSF**

The CSF hemopexin reference range as determined by ELISA was 12.3 to 32.6 μg/mL; the mean concentration was 22.4 μg/mL. There was no sex difference (mean, 21.5 and 24.6 μg/mL in women and men, 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 hemopexin (Q<sub>hemopexin</sub>) divided by the CSF/serum ratio of albumin (Q<sub>albumin</sub>). Albumin is a plasma protein that is not synthesized in the 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 hemopexin across the BBB (hemopexin: molecular weight, 68 kDa and molecular size, 36 Å). Q<sub>hemopexin</sub> was significantly greater than Q<sub>albumin</sub> (P<0.0001, Wilcoxon signed-rank test; Figure 1A); the intrathecal index (Q<sub>hemopexin</sub>/Q<sub>albumin</sub>) was 10.5. Thus, the vast majority of hemopexin is produced intrathecally in control individuals, with only about one tenth being derived from the circulation.

**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–hemopexin complexes leads to intracellular deposition of heme’s iron moiety. 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–hemopexin system actively scavenges heme after SAH.

Increased CSF Hemopexin After SAH Is Associated With Poor Outcome

After SAH, CSF hemopexin had a bimodal distribution; in 30% of patients (n=9/30), CSF hemopexin was above the reference range (Figure 2A). In high versus normal CSF hemopexin patients, delayed cerebral ischemia occurred more frequently and neurological outcome 6 months after SAH, as assessed by the modified Rankin Scale (mRS), was poorer (delayed cerebral ischemia: 57% versus 11%; $P=0.028$, Fisher exact test and mean mRS: 5.0 versus 2.4; $P=0.025$, unpaired t test). The difference in Glasgow Outcome Scale 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 hemopexin groups could not be explained by several other factors related to bleed size or severity (Table III in the online-only Data Supplement). Although more women than men had a high CSF hemopexin, this was not significant, and CSF hemopexin was not significantly different between the sexes (25.2 and 23.3 μg/mL in women and men, respectively; $P=0.699$, Mann–Whitney test). Overall, this indicates that CSF hemopexin 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 7-fold increase in CSF hemopexin (Table III in the online-only Data Supplement) could be blood or brain derived. The hemopexin intrathecal index was significantly raised in high CSF hemopexin patients (4-fold; $P<0.001$, unpaired t test), indicating partial intracranial origin (Figure 2B). The blood-derived fraction could have 2 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 $Q_{albumin}$; 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 hemopexin group as evidenced by a raised $Q_{albumin}$ (2-fold; $P=0.033$, unpaired t test; Figure 2C). Hence, the predominant source of increased CSF hemopexin associated with poor outcome was intrathecal, with some derived from the circulation.

Saturation of Heme–Hemopexin Uptake After SAH

The high CSF hemopexin suggested saturation of heme–hemopexin uptake. To look for this, SAH CSF was examined for the presence of heme–hemopexin complexes. Sufficient CSF from 1 patient in clinical study 1 was available for ultra-high performance liquid chromatography analysis to detect bound heme; in this patient, who had a high CSF hemopexin level (98.5 μg/mL), a substantial amount of heme was bound to hemopexin and albumin (Figure 3A, inset; Figure I in the online-only Data Supplement, peak at 4.5 minutes). In 7 other patients with high-grade SAH, with repeated CSF sampling (clinical study 2), bound heme was found at all time points, confirming rapid saturation of heme–hemopexin uptake, up to at least day 13 after SAH (Figure 3A).

Figure 2. A–C, Cerebrospinal fluid (CSF) hemopexin (Hpx) from controls and patients with subarachnoid hemorrhage (SAH). A, Delayed cerebral ischemia (DCI), Glasgow Outcome Scale (GOS), and modified Rankin Scale (mRS) in high vs normal CSF Hpx SAH patients. B and C, Hpx intrathecal index and albumin quotient of high vs normal CSF Hpx SAH patients.
Free Heme After SAH
In the same 7 patients from clinical study 2, unbound heme was assayed by performing ultrahigh performance liquid chromatography before and after adding recombinant hemopexin 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 preincubated with recombinant hemopexin, 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 hemopexin after SAH (mean, 0.72 mg/mL in controls versus 0.55 mg/mL after SAH; P=0.002, unpaired t test; Figure 3C). This decrease was confined to patients with the highest Qalbumin, 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 characterize the intrathecal CD91–hemopexin system in control individuals and after SAH. Intrathecal production is likely to be the main source of CSF hemopexin, with only about one tenth being derived from the circulation. Hemopexin is known to be produced within the brain, and CD91 is not expressed by cerebral endothelium5,8, so, receptor-mediated transport of hemopexin into the brain is unlikely but cannot be excluded. Still, CSF hemopexin was 10-fold lower compared with serum, suggesting that the brain has a comparatively lower heme-binding capacity.

After SAH, the CD91–hemopexin scavenging system is active in the brain because CD91 significantly correlated with iron deposition. CSF hemopexin had a bimodal pattern after SAH. Despite similar bleed size and severity, high CSF hemopexin patients had a poorer outcome, with higher delayed cerebral ischemia rates and higher mRS scores. This needs replication because CSF hemopexin may be of clinical utility as a prognostic marker. The Glasgow Outcome Scale was not significantly different between individuals with normal and high CSF hemopexin; however, such a discrepancy between Glasgow Outcome Scale and mRS has been noted after SAH before 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, whereas Glasgow Outcome Scale was developed as a scale for use in traumatic head injury.

The elevation of CSF hemopexin, and its relationship with outcome, is intriguing, and the mechanism is not clear. Hemopexin has been reported to be neuroprotective,10 and therefore, CSF hemopexin is not assumed to be toxic.

Figure 3. A and B, Bound and free heme at day 0 up to 8 to 13 days post ictus. Inset shows ultrahigh performance liquid chromatographic analysis of a high hemopexin (Hpx) cerebrospinal fluid (CSF): a substantial peak for Hpx/albumin-bound heme is observed at 4.5 minutes (arrow). C and D, Heme efflux to the periphery after subarachnoid hemorrhage (SAH). C, Serum Hpx in controls and patients with SAH. D, Variation of serum Hpx level with albumin quotient.
However, it is also possible that when CSF hemopexin 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 neurons/glia. Another potential explanation is that events after SAH, such as saturation of heme–hemopexin uptake or inflammation, result in high CSF hemopexin and poor outcome, which are, therefore, indirectly associated. Clearly, further mechanistic studies are needed.

CSF hemopexin had 2 sources: (1) accumulation from an intrathecal origin (as indicated by a 3-fold rise in the hemopexin intrathecal index) and (2) increased transfer from the circulation (supported by a 2-fold higher $Q_{\text{brain}}$). Accumulation of brain-derived hemopexin 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 because the hemopexin promoter is interleukin-6 responsive, and interleukin-6 levels are elevated in SAH CSF. Decreased CD91-mediated scavenging may be because of plateauing in heme–hemopexin uptake because heme and hemopexin were detected simultaneously in the CSF. Also, because CD91 has multiple ligands, it is possible that there is competition for CD91 from other ligands, such as ApoE. Interestingly, CSF ApoE is known to decrease after SAH, and low CSF ApoE associates with a poor outcome, similar to patients with high CSF hemopexin in this study.

Acknowledgments

We thank Ardalan Zolnourian, Southampton General Hospital, for the help with Table II in the online-only Data Supplement.

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Disclosures

Galea and D. Bulters were supported to study hemopexin, and A.I. Okemefuna is employed by Bio Products Laboratory Limited.

References

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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

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<th>SAH</th>
<th>Controls</th>
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<tr>
<td>Number</td>
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<td>20</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.1 ± 9.6</td>
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<td>Sex (% female)</td>
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<tr>
<td>Presentation Glasgow Coma Score</td>
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<tr>
<td>Fisher grade</td>
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<td>WFNS score</td>
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<tr>
<td>DCI: clinical evidence (%)</td>
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<td></td>
</tr>
<tr>
<td>DCI: CT evidence (%)</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>DCI: clinical &amp; CT evidence (%)</td>
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<tr>
<td>6 month Glasgow Outcome Score</td>
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<tr>
<td>6 month Modified Rankin Score</td>
<td>3.0 ± 2.5</td>
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<tr>
<td>Days post-ictus when sample taken</td>
<td>3.9 ± 2.6</td>
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</tr>
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Values represent mean ± standard deviation where applicable. Data available for 26\textsuperscript{a}, 28\textsuperscript{b} and 25\textsuperscript{c} patients. Abbreviations: CT: Computed Tomography; DCI: Delayed Cerebral Ischaemia; WFNS: World Federation of Neurological Surgeons

Table II Demographics of clinical study 2

<p>| | |</p>
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<tr>
<td>Number</td>
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<tr>
<td>Age in years</td>
<td>59.9 ± 17.8</td>
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<td>Presentation Glasgow Coma Score</td>
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<tr>
<td>Fisher grade</td>
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<tr>
<td>DCI: CT evidence</td>
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<tr>
<td>DCI: clinical &amp; CT evidence</td>
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<tr>
<td>3 month Modified Rankin Score</td>
<td>2.2 ± 1.5</td>
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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

<table>
<thead>
<tr>
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<th>Normal CSF Hpx</th>
<th>High CSF Hpx</th>
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<tbody>
<tr>
<td>Number of cases</td>
<td>21</td>
<td>9</td>
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<tr>
<td>CSF Hpx concentration (µg/ml)</td>
<td>18.3 ± 8.0</td>
<td>133.8 ± 42.2</td>
<td>1.4 x 10^{-7}*</td>
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<tr>
<td>DCI: clinical evidence (%)(^a)</td>
<td>11</td>
<td>57</td>
<td>0.028*</td>
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<tr>
<td>DCI: CT evidence (%)(^a)</td>
<td>11</td>
<td>57</td>
<td>0.028*</td>
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<tr>
<td>DCI: clinical &amp; CT evidence (%)(^a)</td>
<td>21</td>
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<tr>
<td>6 month Modified Rankin Score(^b)</td>
<td>2.4 ± 2.2</td>
<td>5.0 ± 2.4</td>
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<tr>
<td>6 month Glasgow Outcome Score(^c)</td>
<td>3.9 ± 1.5</td>
<td>2.5 ± 1.6</td>
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<td>Presentation GCS</td>
<td>11.7 ± 3.6</td>
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<td>Fisher grade</td>
<td>3.6 ± 0.8</td>
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<td>WFNS score</td>
<td>2.7 ± 1.5</td>
<td>3.3 ± 1.6</td>
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<td>Days post-ictus sample taken</td>
<td>4.4 ± 2.6</td>
<td>2.6 ± 2.2</td>
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<td>CSF Hb concentration (µmol/l)(^d)</td>
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<td>0.20 ± 0.18</td>
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<td>Blood clot: mean voxel radiodensity(^e)</td>
<td>103.1 ± 11.1</td>
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<tr>
<td>Age in years</td>
<td>51.1 ± 9.0</td>
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<td>Sex (% female)</td>
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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. 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. 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, 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.
References

