Supplemental Methods

Scoring of Magnetic resonance imaging scans

MRI scans were assessed by two fellowship trained radiologists (one a neuroradiologist). This was on the basis of a pre-determined, pre-specified 8 point score based on the radiological appearance of the cerebrum: 1 – marked atrophy; 2 – mild atrophy; 3 – normal (no atrophy and no increased volume); 4 – subtle increased volume; 5 – mild increased volume; 6 – obvious but moderately increased volume; 7 – substantial volume increase with effacement of the sulci and cisterna but without herniation; 8 – as 7 but with evidence of herniation. This was then collapsed to a 4-point score: 1) No brain swelling (score 1-3); 2) mild brain swelling (score 4 or 5); 3) moderate brain swelling (6) and 4) severe brain swelling (7 or 8). Decision on a score when the two reviewers had discrepant results was arrived at by a pre-specified system that included a confidence factor assigned by each radiologist in the score given. The system for reviewing the scans has been described in detail in a standalone article[12].

Isolation and purification of *P. falciparum* histones.

P. falciparum histones (H2A/H2B, H3 and H4) were purified from late trophozoite and schizont stage IT4var16 IE using a Histone Purification Kit (Active Motif). Parasites were isolated by 0.2% saponin lysis, and washed in cold PBS and twice with 25 mM TRIS-HCl, 1 mM EDTA, 0.2% NP40, pH 7.8 to remove haemoglobin and membrane fragments. After a wash with 25 mM TRIS-HCl, 1 mM EDTA, pH 7.8, histones were extracted overnight at 4 °C, neutralised, purified by chromatography and precipitated with 4% perchloric acid. The histone pellet was washed twice with 4% perchloric acid, twice with acetone containing 0.2% HCl, twice with acetone, air dried and resuspended in 10 mM TRIS, 1 mM EDTA pH 8.0. Protein concentration was determined by Bio-Rad Protein Assay, using both bovine serum albumin and purified calf histones as standards, and purity examined by SDS-PAGE and Coomassie staining (>95% pure; Supplemental figure S3)

Assay to quantify histones in patient serum

Western blotting was used to quantify circulating histones in control and patient serum samples. Serum was mixed with SDS lysis buffer (1% SDS, 120 mM Tris, 25 mM EDTA, pH 6.8) and heated at 100°C for 10 minutes. Proteins were then separated on 15% SDS-PAGE gels and transferred onto Immobilon-P PVDF membranes (Immobilon, UK). Following blocking, membranes were incubated with primary antibody against histone H3 (abcam; 1:2000). The membrane was then washed in TBS-T and incubated with a secondary anti-rabbit antibody-tagged with horseradish peroxidise (HRP) (Santa Cruz; 1:10,000). The membrane was then washed in TBS-T. Bands were visualized by chemiluminescent detection using G box gel imaging system (Syngene) and software GeneSnap (Syngene). Densitometry was performed using GeneTools software (Syngene) and histones were quantified using recombinant Histone H3 (Supplemental Figure 3).

Mass spectrometry sample preparation.

Purified *P. falciparum* and human histones (New England Biolabs) (6µg), normal serum, histone spiked serum and CM patient serum were separated by 15% SDS-PAGE and stained with Coomassie brilliant blue. The excised gel slices (<35kDa) from SDS-PAGE, were cut into $1mm^3$ plugs, transferred to a microtube and fully de-stained using 25mM Ambic alternately with Ambic/MeCN (2:1). Cysteine reduction was performed by adding 100µL DTT solution (1.5mg/mL) and incubated at 60°C for 60 min. Samples were centrifuged and the supernatant was discarded. Alkylation was performed by the addition of 100µL iodoacetamide (10mg/mL) for 45 min (protected from light). Samples were centrifuged and the supernatant discarded. Gel plugs were then washed with Ambic (25mM) for 15min at 37°C. To fully dehydrate the gel plugs, samples were washed with MeCN. In-gel digestion was performed by adding 100µL of trypsin (12.5ng/µL in 25mM Ambic) to each sample with overnight incubation at 37°C, and reactions terminated by the addition of 10µL formic acid (1% final concentration). The solutions surrounding the gel plugs (containing the tryptic peptides) were retained for analysis. To extract additional peptides from the gel plugs, a further incubation with a

solution containing water:MeCN:FA (50:49:1) and then MeCN:FA (80:19:1) was performed. Finally, solutions were pooled and dried to a 10µL solution.

Liquid Chromatography-mass spectrometry analysis.

Analysis was performed using an Ultimate 3000 RSLC™ nano system (Thermo Scientific, Hemel Hempstead), coupled to a QExactive-Hf™ mass spectrometer (Thermo Scientific). Samples were loaded onto a trapping column (Thermo Scientific, PepMap100, C18, 300 µm X 5 mm), using partial loop injection, for seven minutes at a flow rate of 9 µL/min with 0.1% (v/v) FA. Samples were then resolved on the analytical column (Easy-Spray C18 75 µm x 500 mm 2 µm column) using a gradient of 97% A (0.1% formic acid) 3% B (99.9% ACN 0.1% formic acid) to 60% A 40% B over 15 min at a flow rate of 300 nL min^{-1}. The data-dependent program used for data acquisition consisted of a 70,000 resolution full-scan MS scan (AGC set to 1 x 10^6 ions, with a maximum fill time of 20ms) the 10 most abundant peaks were selected for MS/MS using a 35,000 resolution scan (AGC set to 1 x 10^5 ions with a maximum fill time of 100ms) with an ion-selection window of 3 m/z and a normalized collision energy of 28. To avoid repeated selection of peptides for MS/MS the program used a 15 second dynamic exclusion window. Sequence alignment was performed in PEAKs software (v8.5) against both *P. falciparum* and *Homo sapiens* databases. Once species-specific peptides were identified they were further verified using Skyline analysis software for quantification (comparisons between the specific amino acid sequences of *P. falciparum* and *Homo sapiens* histone proteins illustrated in Supplemental figure S6).

To establish the sensitivity and specificity of the assay to detect and distinguish between Plasmodial and human origins we performed spiking experiments using purified histones. We used different concentration and ratios of Plasmodial and human histones in normal serum. The lowest detected concentration of individual histones (H1, H2A, H2B, H3 and H4) in this assay was 5 µg/ml, which would equate to approximately 20 µg/ml total circulating histones. Hence the test is specific but does lack

sensitivity. We then assessed by Mass Spectrometry 10 cases selected at random from all those with levels above 20 μg/ml and with sufficient remaining serum for the assay (Range 24 μg/ml – 884 μg/ml; Median 69.9 μg/ml).

Immunohistochemistry

Cortical sections (4μm in thickness) were stained for histones and fibrinogen. Heat-induced antigen retrieval using a pressure cooker and citrate buffer (pH 6.0) was performed prior to overnight incubation with primary antibodies (4°C): rabbit anti-histone H3 (ab1791, Abcam; 5μg/ml); goat anti-Fibrinogen (PA1-26809, Thermofisher; 18μg/ml). Bound primary antibody was detected using alkaline phosphatase and red staining for detecting histone staining (Vector Red, Vectorshield) and HRP and 3,3'-Diaminobenzidine (DAB) for detecting fibrinogen staining (Envision plus, Dako). Negative controls without primary antibody were used for all samples to confirm specificity.

Assessment of Endothelial Barrier

Permeability of confluent HBMEC monolayers was analyzed in a dual-chamber system (0.4 μM pore size; Millipore). HBMEC were treated with normal serum or patient serum (diluted 1:1 with PBS) for 1 hour and replaced with horse radish peroxidase (HRP)-containing media. Leaked HRP over 1 hour was determined using TMB substrate (Thermofisher) on a microplate reader (450nm). Permeability was expressed as a fold change compared to monolayers treated with pooled normal serum from healthy UK donors [RETH000685].We used 3- 8 biological replicates for all experiments.

For transendothelial electrical resistance experiments primary human brain endothelial cells (HBMEC, Cell Systems) were grown in Endothelial Cell Growth Medium 2 in the presence of 2% FCS (EGM2, Promocell). Endothelial barrier impedance was measured using xCELLigence system (ACEA Biosciences) on 16 well E-plates coated with bovine fibronectin (10 μg/ml). HBMEC were seeded at 15,000 cell/well and grown overnight until confluence was reached, the culture medium was

exchanged for Opti-MEM® medium supplemented with 1% FCS and maintained for 2 h before treatment with *P. falciparum* histones at 25, 50 and 100 µg/ml or histones pre-incubated with +/- non-anticoagulant N-acetyl heparin (200 µg/ml; Sigma) for 15 min before application to HBMEC. Plasmodial histones were also pre-incubated with 200µg/ml of anti-histone single chain variable Fragment (or ahscFv coupled magnetic beads (Pierce™NHS-Activated Magnetic Beads, Thermofisher) before application to HBMEC. Controls included media-only and heparin only treatment. As baseline for the experiments medium-only control was used. Barrier function was defined as changes occurring in the cell impendence after normalization to the media-only control at the time of treatment application. Cell index (CI) slope was used to measure the inclination of the CI curve in defined time range characterized by active decrease of CI. Higher negative slopes denote faster kinetics of impendence change.

	Classification	Diagnosis	% Vessels with High Sequestration	% Vessels with Strong Histone staining	% Vessels with Leak
74	CM1	Cerebral malaria	67.1%	17.1%	2.9%
79	CM1	Cerebral malaria	73.3%	6.7%	2.2%
84	CM1	Cerebral malaria	60.0%	0.0%	0.0%
97	CM1	Cerebral malaria	82.9%	4.3%	1.4%
99	CM1	Cerebral malaria	22.2%	4.4%	0.0%
100	CM1	Cerebral malaria	3.3%	0.0%	0.0%
60	CM2	Cerebral malaria	55.7%	1.4%	1.4%
61	CM2	Cerebral malaria	30.0%	2.9%	7.1%
62	CM2	Cerebral malaria	65.7%	1.4%	1.4%
63	CM2	Cerebral malaria	82.9%	1.4%	7.1%
64	CM2	Cerebral malaria	85.6%	2.2%	3.3%
66	CM2	Cerebral malaria	4.3%	0.0%	1.4%
68	CM2	Cerebral malaria	75.7%	0.0%	1.4%
75	CM2	Cerebral malaria	82.9%	24.3%	1.4%
78	CM2	Cerebral malaria	22.9%	5.7%	14.3%
101	CM2	Cerebral malaria	21.4%	10.0%	20.0%
102	CM2	Cerebral malaria	47.8%	1.1%	14.4%
43	CM3	Giant cell myocarditis	0.0%	0.0%	5.6%
49	CM3	Ruptured Arteriovenous malformation	0.0%	4.4%	1.1%
54	CM3	Skull fracture	0.0%	0.0%	10.0%
71	CM3	Subdural/intracerebral hematomas	0.0%	0.0%	4.4%
92	CM3	Left ventricular failure with pulmonary edema	0.0%	0.0%	0.0%
93	CM3	Clinical CM; Diagnosis uncertain	0.0%	0.0%	0.0%
44	Non-CM	Salicylate toxicity - suspected	0.0%	1.1%	4.4%
46	Non-CM	Severe (non-malarial) anaemia	0.0%	0.0%	0.0%
59	Non-CM	Reye's syndrome	0.0%	0.0%	1.4%
65	Non-CM	Reye's syndrome	0.0%	0.0%	0.0%
88	Non-CM	Subdural hematoma, head trauma	0.0%	0.0%	0.0%

Table S1. *Summary of post-mortem cases.* Clinical pathologist's diagnosis at autopsy and proportion of vessels with each of: (1) high sequestration (seq; sequestration involving >50% of vessel lumen); (2) strong histone staining and; (3) leak (fibrinogen staining adjacent to a vessel).

Patient number	Circulating histones (µg/mL)	P. falciparum histones Mass spec; Mean peptide intensity (x10^4)	Human histones Mass spec; Mean peptide intensity (x10^4)	Pf + Human histones combined	Proportion Plasmodial	Proportion Human	PfHRP2 (ng/ml)	Peripheral parasite density (per µl)
2867	37.5	18.0	6.1	24.1	0.75	0.25	3584	95880
2896	69.8	1.8	80.0	81.8	0.02	0.98	7115	1151280
2905	109.3	51.5	33.5	85.0	0.61	0.39	10702	335980
2851	239.4	32.3	4.0	36.3	0.89	0.11	40211	447674
2880	435.1	5.1	11.0	16.1	0.31	0.69	30520	385
2877	25.88	6.2	10.0	16.2	0.38	0.62	5204	165000
2871	70	1.6	6.8	8.4	0.19	0.81	20208	17701
2853	24	15.8	43.8	59.6	0.26	0.74	18447	364
2861	49.68	9.2	4.2	13.4	0.68	0.32	3881	49560
2885	884	50.0	6.0	56.0	0.89	0.11	7250	49280

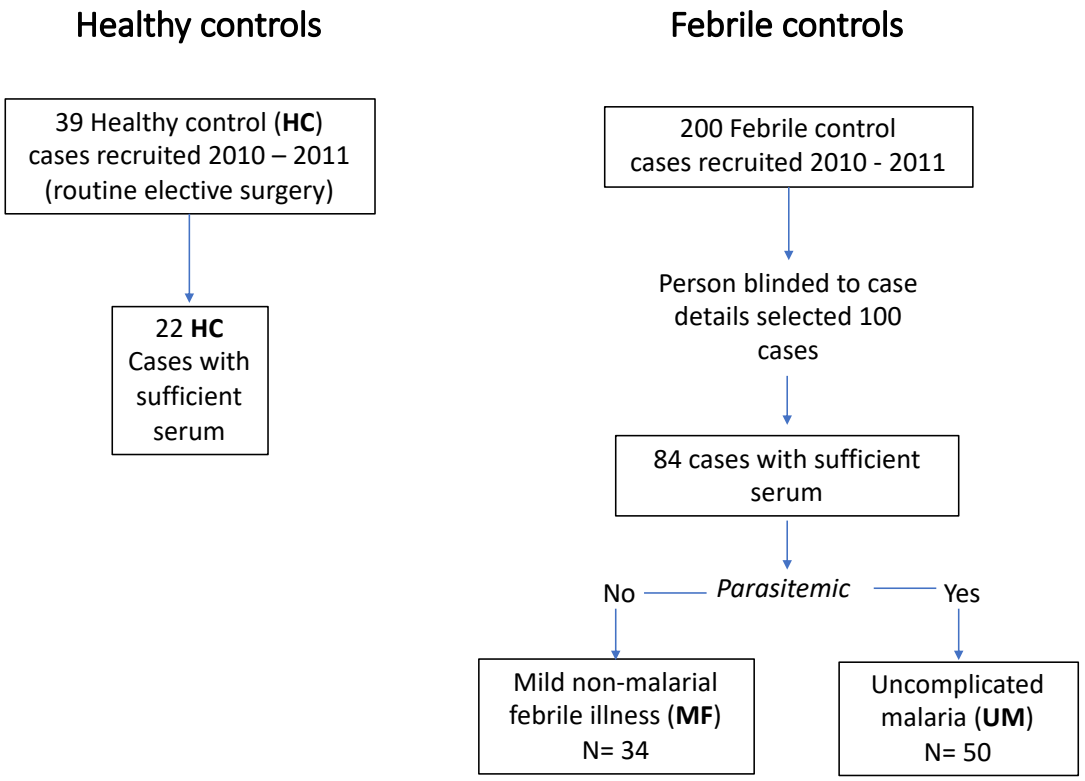

Figure S1: Non-comatose cases included in the analysis

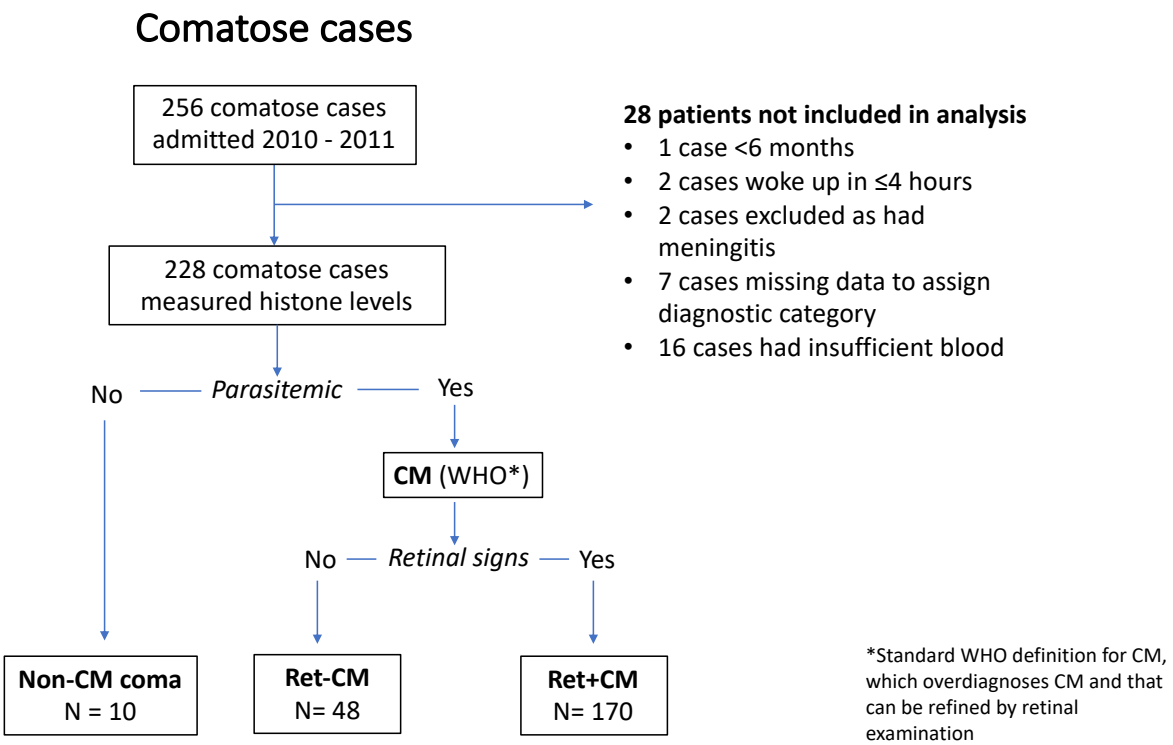

Figure S2: Comatose cases included in the analysis for serum/ plasma samples

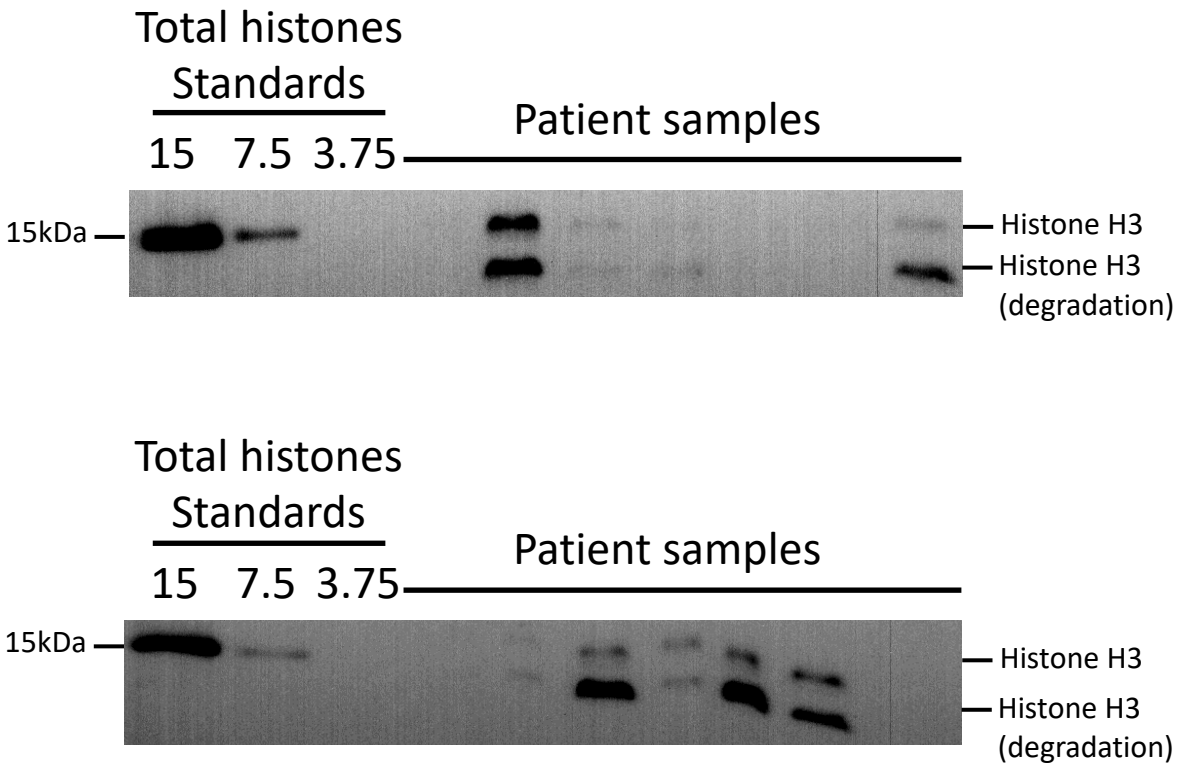

Figure S3. Histone quantification. Circulating histones were quantified in serum from patients by Western blot using purified histones as standards. Typical blots are presented.

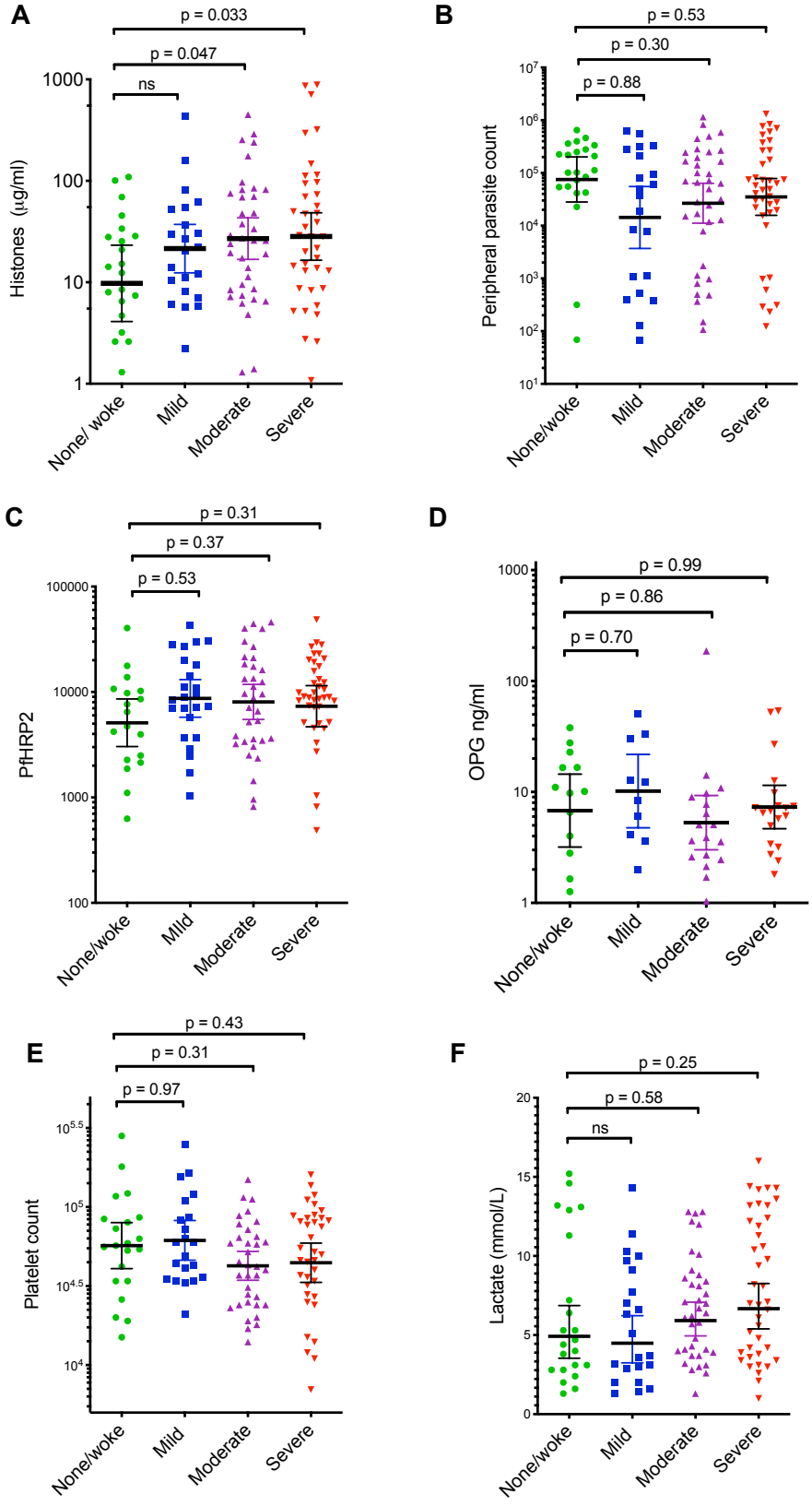

Fig S4. Histones but not other laboratory factors are associated with the degree of brain swelling in CM-pos patients. PfHRP2 = *P. falciparum* histidine rich protein 2; OPG =osteoprotegerin

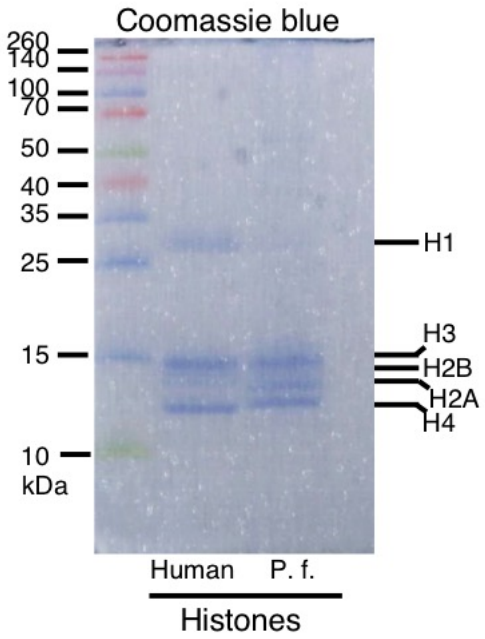

Figure S5. **Gel showing purified Plasmodium falciparum (P. f.) and human histones.** Different core histones (H2A, H2B, H3, H4) are identified by size.

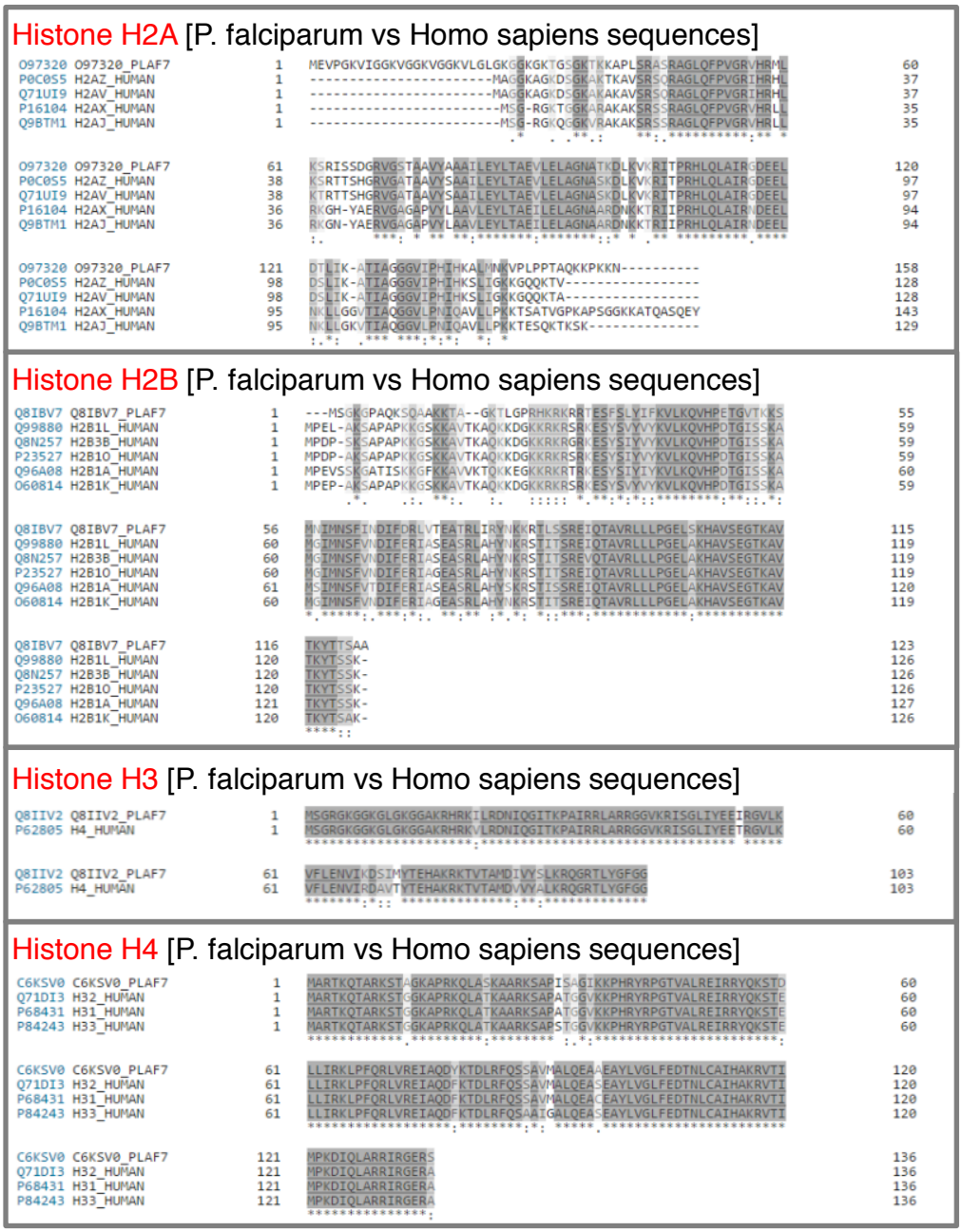

Fig S6. Alignment of Homo sapiens and P. falciparum histones. Amino acid sequences of individual histone variant proteins (H2A, H2B, H3 and H4) were compared between *Homo sapiens* and *P. falciparum*. Using these data, we were able to identify heterologous (species-specific) histone peptide sequences (including protein ID numbers) for further downstream analysis. Dark grey = homologous amino acids; light grey and clear = heterologous amino acids.

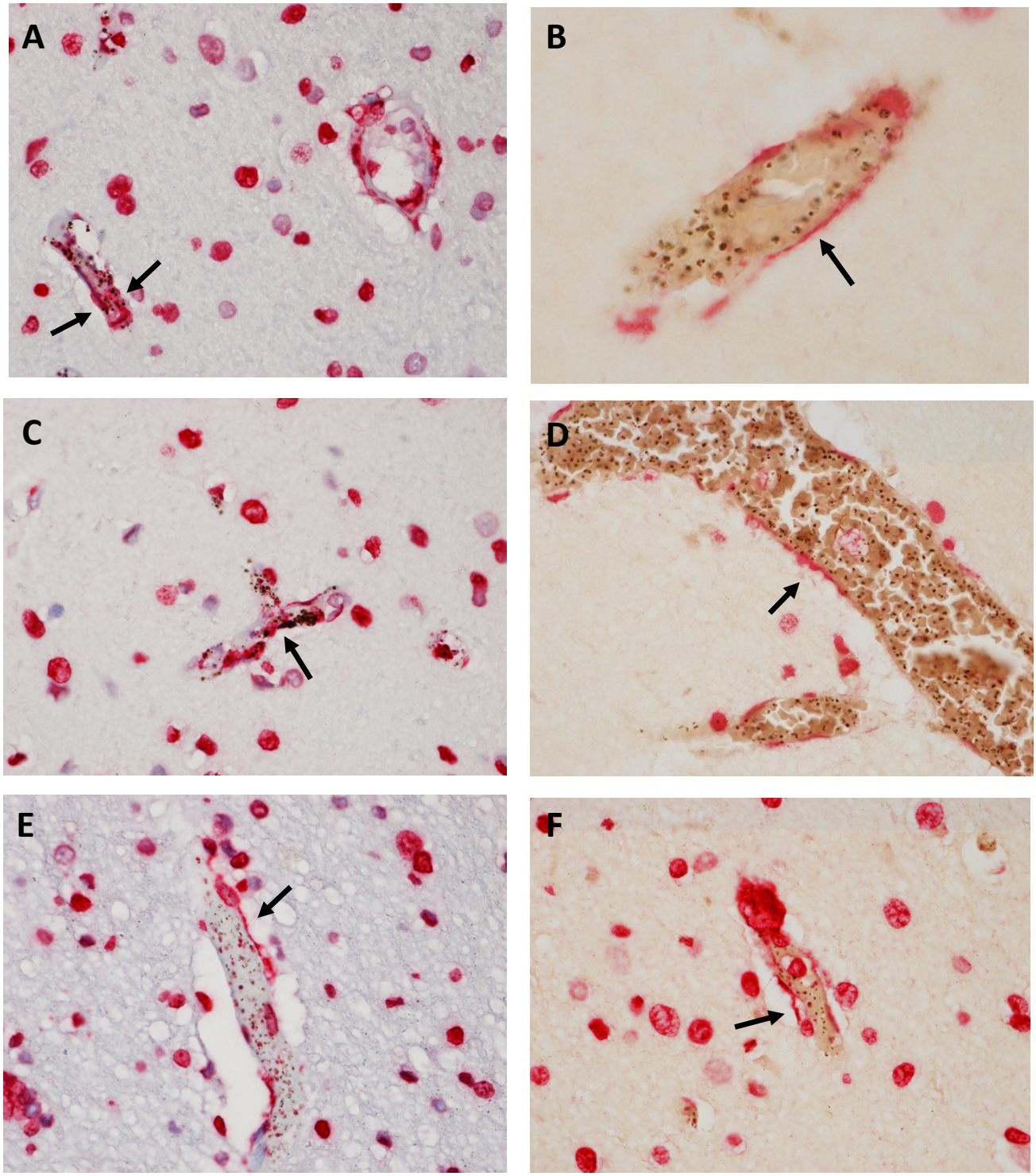

Figure S7. Vessels from definitive CM cases showing red histone staining in nuclei and red luminal histone staining (black arrows) in significantly parasitized vessels. Red staining is to anti-H3 antibody using Vector Red (A-F) and brown staining (B,D,F) is to anti-fibrinogen antibody using DAB (3,3'-diaminobenzidine). Images were acquired at x600 using an oil immersion lens. Image B is enlarged to show luminal histone staining in a smaller vessel.

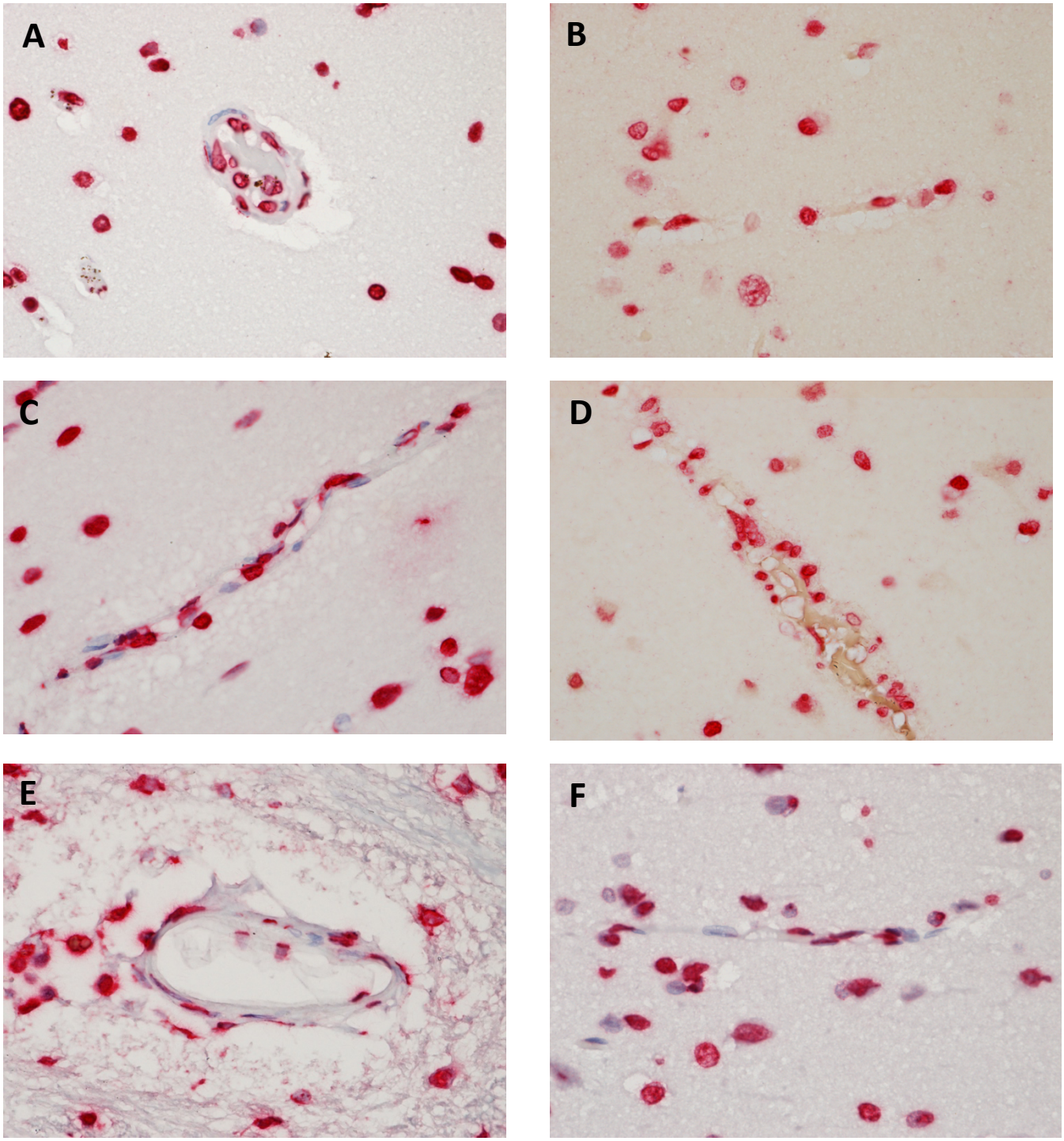

Figure S8. Vessels from brain tissue from fatal parasitemic non-CM cases (A – D) aparasitemic comatose cases (E and F) showing clear (red) histone staining in nuclei but no histone membrane staining. Red staining is to anti-H3 antibody using Vector Red and brown staining (B,D) is to anti-fibrinogen antibody using DAB (3,3'-diaminobenzidine). Images were acquired at x600 using an oil immersion lens.

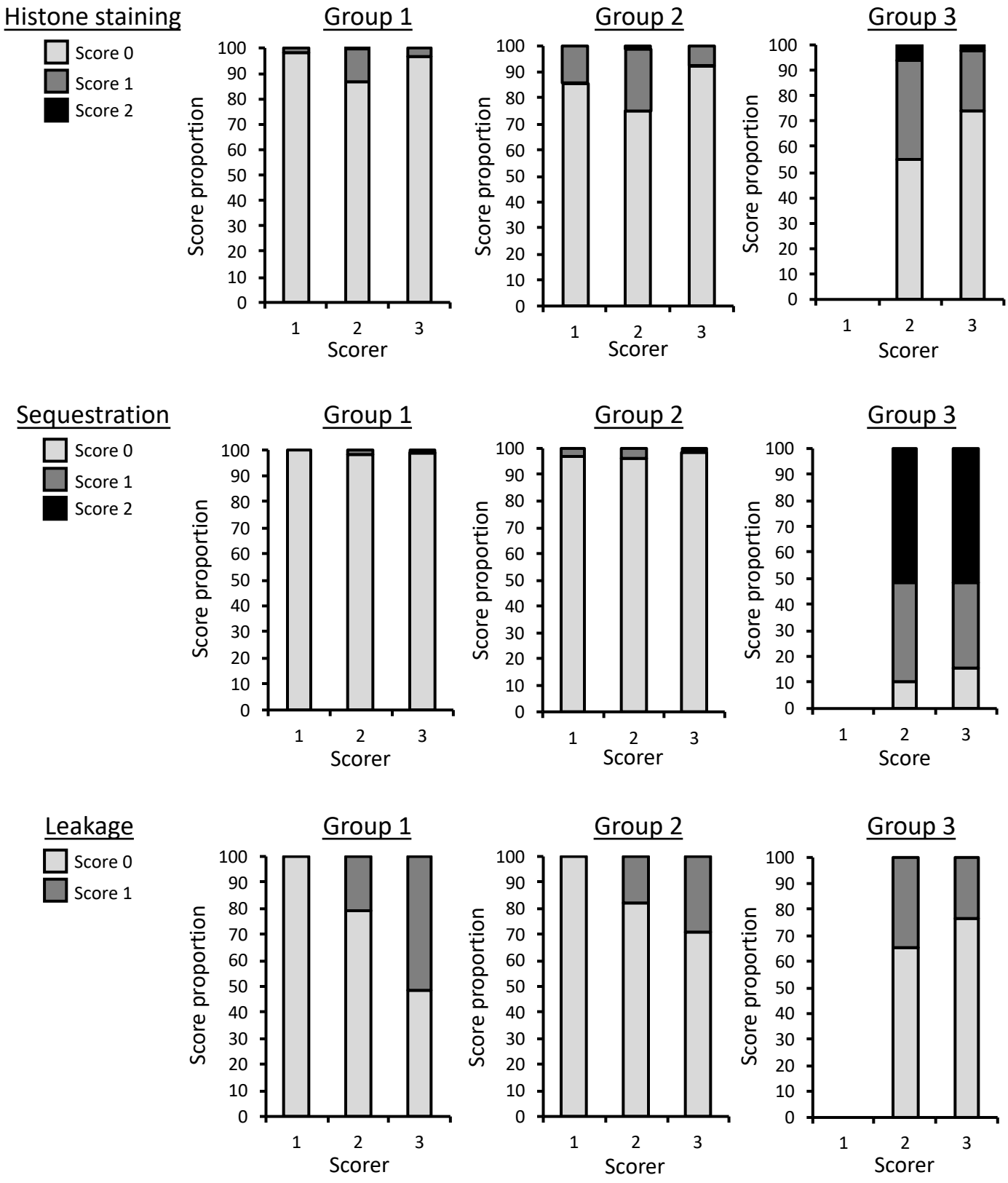

Figure S9. *Scorer comparison.* Histograms display the percentage of vessels for which each scorer assigned each score for the 3 scored modalities (Histone staining, sequestration and Leakage) segregated by group. Histone membrane staining for each vessel was scored as absent (0); weak (+) or strong (++). IE sequestration for each vessel was scored as: negative (0); positive but <50% of the vessel lumen (+) or >50% of the vessel lumen (++, high). Fibrinogen extravasation as a marker of leak was scored for each vessel as absent or present.

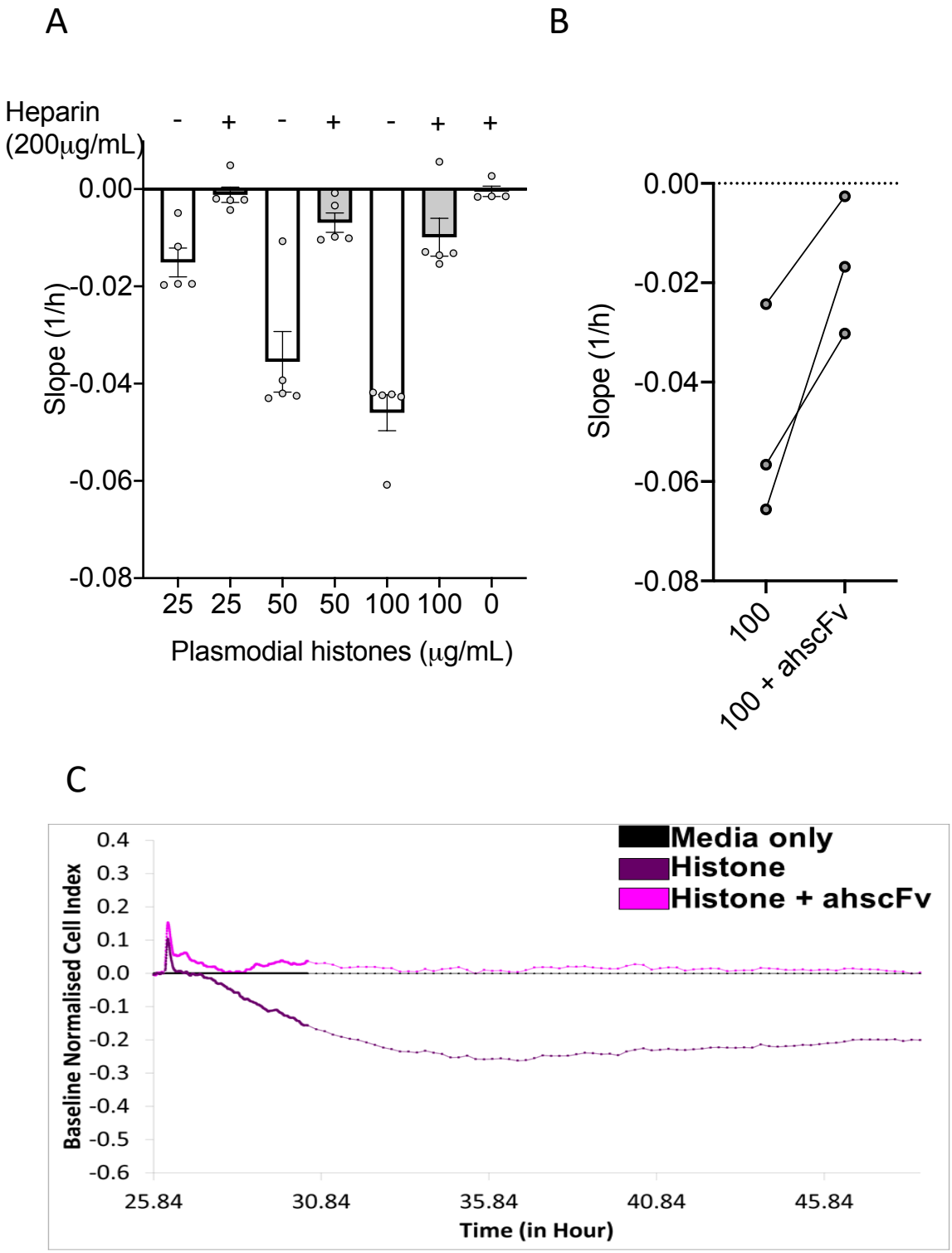

Figure S10. The effect of Plasmodial histones on the endothelial barrier function of human brain microvascular endothelial cells (HBMEC) using trans endothelial electrical resistance (TEER). Cell Index was normalised to the point immediately before treatment application. Data are presented as Cell index (CI) slope to measure the inclination of the Cell Index curve in defined time range characterized by active decrease of CI. **A)** The effect of the Plasmodial histones at 25, 50 and 100 µg/ml alone or pre-incubated with non-anticoagulant N-acetyl heparin (200 µg/ml; Sigma) for 15 min before application to HBMEC. **B)** The effect of pre-treatment of the Plasmodial histones (100 µg/ml) with 200µg/ml of anti-histone single chain variable Fragment (ahscFv) before application to HBMEC. **C)** Baseline normalised Cell index traces for the effect of pre-treatment of the Plasmodial histones (100 µg/ml) with 200µg/ml of anti-histone single chain variable Fragment (ahscFv).

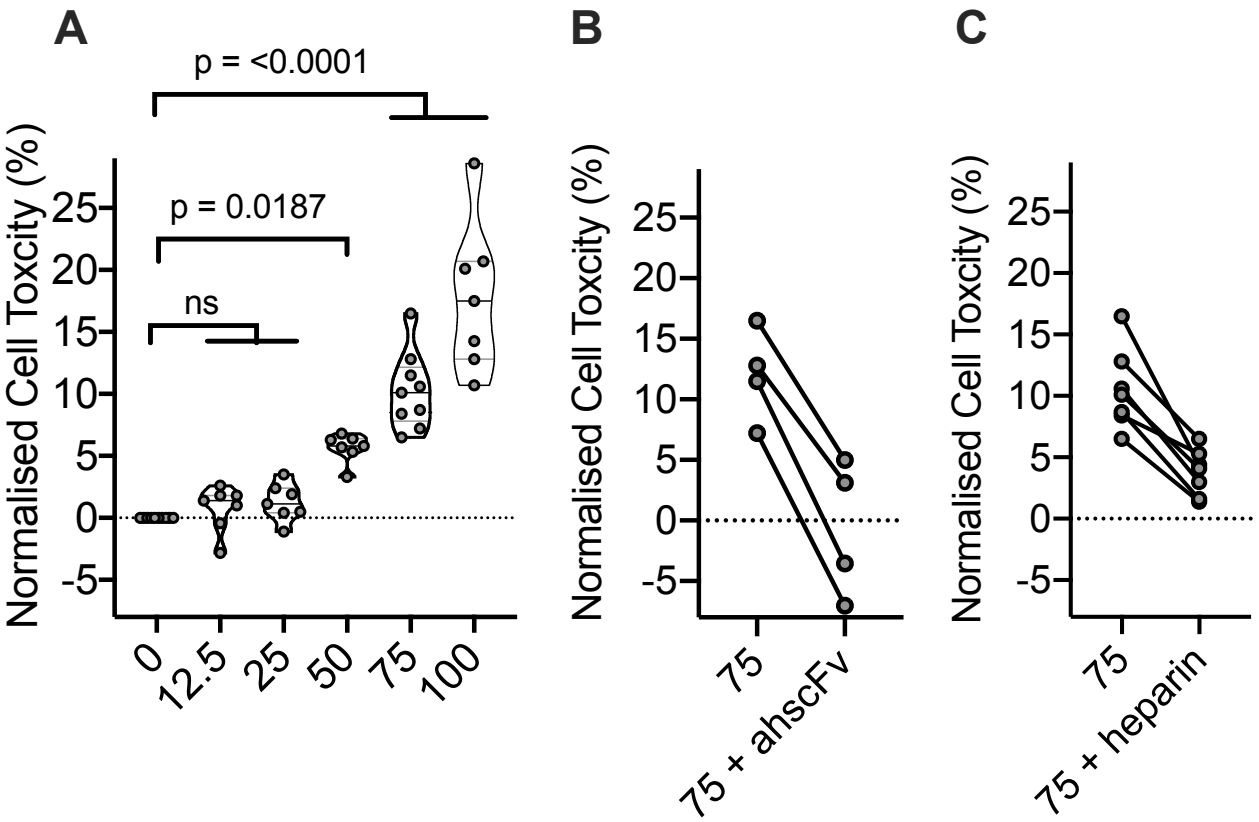

Figure S11. *Plasmodial histones cause toxicity, reversed by ahscFv and non-anticoagulant heparin*

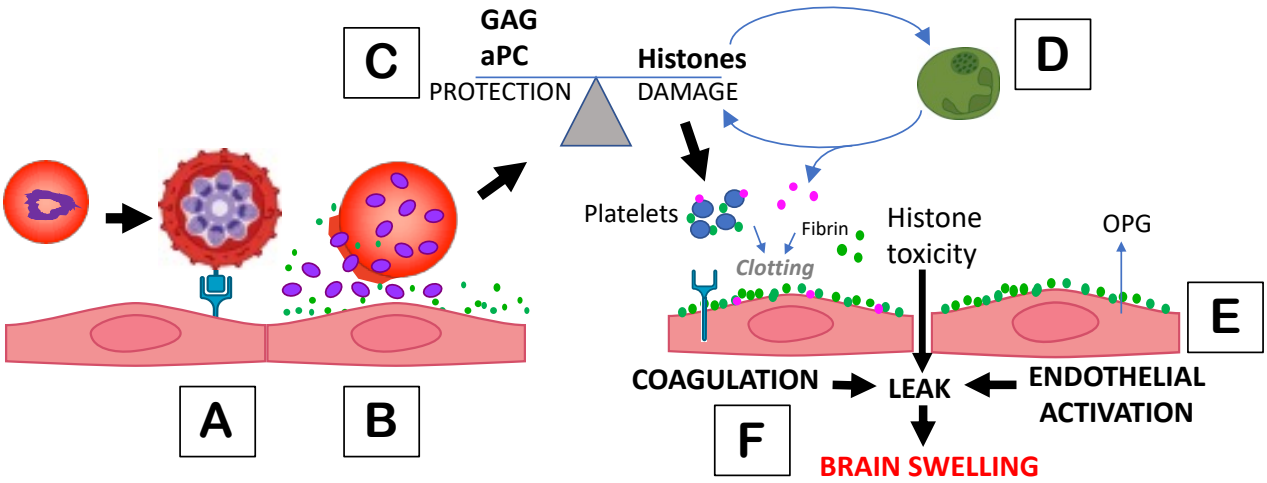

Figure S12. Proposed mechanism of Plasmodial histone-induced brain endothelial disruption. **A.** Infected erythrocytes (IE) stick and sequester to endothelial cells (EC). Replication increases nuclear material (including histones) by an order of magnitude **B.** IE- rupture concentrates histone release to the EC surface **C.** Glycosaminoglycans (GAG) or activated protein C (aPC) buffer histones until local histone production outstrips GAG and aPC production **D.** Histones from damaged host cells act as amplifiers; including from NETs which are activated by heme in IE[44] and also by histones[20] (Neutrophil in green, host histones - pink). **E.** *Coagulation*: Histones cleave thrombin promoting fibrin production (directly) and also cause platelet aggregation, together causing clotting. *Endothelial Activation*: by histones leads to release of osteoprotegerin (OPG). *Endothelial damage*: Histones incorporate into EC membranes and cause calcium influx leading to EC damage and cause loss of tight junctions leading to BBB breakdown and **F.** leak of fluid into the brain, leading to brain swelling.