**Neurofilament light predicts neurological outcome after subarachnoid haemorrhage**

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## Abstract

To improve outcome prediction following subarachnoid haemorrhage (SAH), we sought a biomarker integrating early brain injury and multiple secondary pathological processes in a prospective study of 42 non-traumatic SAH patients and 19 control individuals. Neurofilament light (NF-L) was elevated in CSF and serum following SAH. CSF and serum NF-L on days 1-3 post-SAH strongly predicted modified Rankin score at six months, independent of WFNS. NF-L from day 4 onwards also had a profound impact on outcome. To link NF-L to a SAH-specific pathological process, we investigated NF-L's relationship with extracellular haemoglobin. Most CSF haemoglobin was not complexed with haptoglobin, yet was able to be bound by exogenous haptoglobin i.e. haemoglobin was scavengeable. CSF scavengeable haemoglobin was strongly predictive of subsequent CSF NF-L. Next, we investigated NF-L efflux from the brain after SAH. Serum and CSF NF-L correlated positively. The serum/CSF NF-L ratio was lower in SAH versus controls, in keeping with glymphatic efflux dysfunction after SAH. CSF/serum albumin ratio was increased following SAH versus controls. The serum/CSF NF-L ratio correlated negatively with the CSF/serum albumin ratio, indicating that transfer of the two proteins across the blood-brain interface is dissociated. In summary, NF-L is a strong predictive marker for SAH clinical outcome, adding value to WFNS, and is a promising surrogate endpoint in clinical trials.

## Introduction

Subarachnoid haemorrhage leads to an increase in intracranial pressure, transient cerebral ischemia, and a subsequent prolonged multifactorial insult including neurotoxicity from haemoglobin released from the blood clot, delayed cerebral ischaemia, and inflammation (Macdonald, 2014). To predict long-term outcome in this patient population, it will be necessary to understand the contributions of early brain injury and subsequent pathological events, particularly from the prolonged exposure of the brain to haemoglobin and inflammatory molecules. Clinically, early brain injury can be assessed using the World Federation of Neurosurgical Societies (WFNS) score (Teasdale *et al.*, 1988), which can also partially predict long-term neurological outcome. In the largest study of outcome prediction to date, a number of predictors were tested including age, WFNS grade, premorbid history of hypertension, Fisher scale, aneurysm size, aneurysm location, and treatment modality (Jaja *et al.*, 2018). WFNS only explained a minor proportion of variance in outcome, and the contribution of the other predictors was substantially lower, by one or two orders of magnitude.

In trying to explain a larger proportion of outcome variance, we took advantage of neurofilament light (NF-L), an intracellular neuronal protein released during damage to neurones. NF-L can be detected in both CSF and serum and is a well-established biomarker for neuronal injury in neurodegenerative and neuroinflammatory conditions (Simren *et al.*, 2020). Since NF-L release can integrate neuronal damage from early brain injury as well as a number of delayed secondary pathological processes occurring subsequent to early brain injury, we hypothesized that NF-L can predict outcome after SAH, independent of WFNS.

## Methods

### Sample collection

This study had ethical (National Research Ethics Committee approval numbers 11/SC/0204 and 12/SC/0666) and institutional (ERGO 41084 and ERGO 10492) approvals. Paired CSF and serum were collected daily on the first three days and on alternate days thereafter over a 14 day period post-ictus in 44 patients after original Fisher grade 3-4 (Fisher *et al.*, 1980) non-traumatic SAH with an external ventricular drain *in situ*, until this was removed. WFNS ranged between 1 and 5. Only 42 patients contributed to this study due to

early removal of the EVD. Fresh CSF was collected after discarding a CSF volume equal to the dead space of the tubing. Control CSF was from 19 individuals undergoing lumbar puncture for non-inflammatory / haemorrhagic / degenerative neurological conditions, subsequently found to have normal CSF (glucose, protein, cell count and microbiological assessment). CSF was centrifuged at 1500 rcf for 10 min at 21°C and frozen within 1 h of sampling. Blood samples were routinely taken on equivalent days to CSF drainage. SAH patients were followed up at 6 months to determine clinical outcome as assessed with the modified Rankin Scale (mRS) (Quinn *et al.*, 2009) by researchers trained in using this scale.

## CSF NF-L assay

CSF NF-L was measured by enzyme-linked immunosorbent assay (UmanDiagnostics, Umeå, Sweden). Briefly, samples were thawed at 21°C, and centrifuged at 1750 RCF for five minutes at 21°C. Samples were diluted 1:2 with sample diluent and added in duplicate to microplate wells coated with a monoclonal capture antibody specific for NF-L. Next, samples were incubated with a biotinylated anti-NF-L monoclonal detection antibody. The detection complex was completed with the addition of horseradish peroxidase-labelled streptavidin and tetramethylbenzidine (TMB) substrate. Sample concentrations were extrapolated from a standard curve, fitted using a 4-parameter logistic algorithm. Intra-assay CVs were 1.8% and inter-assay CVs were 10.9%, calculated according to ISO 5725-2 standards.

## Serum NF-L assay

Serum NF-L was measured by Single molecule array (Simoa) on an HD-1 analyser (Quanterix, Billerica, MA), according to manufacturer's instructions (Lot: 501832). Briefly, samples were thawed at 21°C, vortexed, and centrifuged at 10,000 RCF for five minutes at 21°C. On-board the instrument, samples were diluted 1:4 with sample diluent and bound to paramagnetic beads coated with a capture antibody specific for human NF-L. NF-L bound beads were then incubated with a biotinylated anti-NF-L detection antibody in turn conjugated to streptavidin-β-galactosidase complex that acts as a fluorescent tag. Subsequent hydrolysis reaction with a resorufin β-D-galactopyranoside substrate produces a fluorescent signal proportional to the concentration of NF-L present. Duplicate measurements were taken of each sample. Sample concentrations were extrapolated from a standard curve, fitted using a 4-parameter logistic algorithm. Intra-assay CVs were less than 11%, as determined by two quality controls (low and high concentration) according to ISO 5725-2 standards.

## CSF haemoglobin assay

Ultra-performance liquid chromatography (UPLC) was used to separate CSF components in a tris-saline mobile phase, coupled to absorbance measurement at 415 nm to identify haem-containing species (Garland *et al.*, 2020). Haemoglobin was measured in CSF before and after saturating with Hp (Bio Products Laboratory Limited, Elstree, UK) to measure Hp-scavengeable and Hp-non-scavengeable uncomplexed haemoglobin.

## CSF/serum albumin quotient

Albumin in matched CSF and serum samples was measured using a spectrometric assay run on a Beckman Coulter clinical chemistry analyser. The assay relies on the reaction of albumin with Bromocresol Purple to form a coloured complex; absorbance is measured at 600 nm to obtain an albumin measurement. Lumbar CSF albumin values were corrected by a factor of 2.2 (Weisner and Bernhardt, 1978) to enable comparison with ventricular CSF albumin values.

## Statistical analysis

Statistical analysis and graph preparation were performed using SPSS (v24) and GraphPad Prism (v7.01), with data expressed as median ± inter-quartile, mean ± standard error, or 95% confidence intervals. The NF-L reference range was defined as the mean plus and minus 1.96 standard deviations of the control population log-normal distribution. Area-under-the-curve analysis was undertaken using the trapezoid method, and normalized per day. Normality and heteroscedasticity were routinely determined across all data sets. Where necessary, logarithmic transformation was used to normalize data. Model comparison for multivariate linear and ordinal regressions was performed using the F-test. In regression modelling, WFNS was dichotomized into low grade (i.e. 1-3) and high grade (i.e. 4-5). In ordinal regression, the proportional odds assumption was fulfilled. The Nagelkerke's pseudo R-squared value was used. Alpha ($\alpha$), the probability of a Type I error, was 0.05. Two-tailed hypotheses were considered throughout.

## Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request, subject to institutional agreements and ethical approvals.

## Results

### NF-L rises in both CSF and serum after SAH

Demographic and clinical characteristics are shown in Supplementary Table 1. CSF NF-L exhibited a small peak centred around day 2 (Figure 1A), following which both CSF and serum NF-L concentrations gradually increased over the two week sampling period (Figure 1A). Compared to control NF-L levels, the maximum observed NF-L level in both CSF and serum was significantly greater (Figure 1B). This is despite the fact that CSF NF-L is physiologically lower in ventricular CSF compared to lumbar CSF (Jeppsson *et al.*, 2013). Maximum CSF and serum NF-L were not significantly different between patients undergoing clipping, coiling or supportive management in an analysis of covariance controlling for age and WFNS (p=0.926 and p=0.511 for CSF and serum NF-L respectively, Supplementary Figure 1).

### Early NF-L rise in both CSF and serum predicts neurological outcome after SAH

In view of the NF-L peak at day 2, we first investigated whether early NF-L measurement between days 1 and 3, could explain additional variance in outcome alongside WFNS. Multivariable ordinal logistical regression was conducted, with mRS at six months post-ictus as the dependent variable, and NF-L as predictor, controlling for WFNS. Age, gender, premorbid history of hypertension, Fisher scale, size and location of ruptured aneurysm lumen, and treatment modality were not significant covariates in univariate analysis. High CSF and serum NF-L were defined as higher than the upper limit of the respective control population-based reference ranges. High CSF NF-L significantly predicted a worse mRS (Figure 1C), with an odds ratio of 4.3 (95% CI: 1.1-16.2, p=0.031) for poor outcome. The odds ratio for WFNS was 10.4 (95% CI: 2.2-47.8, p=0.003). CSF NF-L and WFNS independently explained six month mRS, such that CSF NF-L improved model fit compared to WFNS alone (pseudo R-squared values: WFNS: 0.306, CSF NF-L: 0.179, WFNS + NF-L: 0.399, logit link function).

Similarly, high serum NF-L significantly predicted a poor neurological outcome at 6 months post-ictus (Figure 1D), with an odds ratio of 2.3 (95% CI: 0.6-6.6; p = 0.04). The odds ratio for WFNS was 3.2 (95% CI: 0.9-9.9, p=0.015). Serum NF-L and WFNS independently explained six month mRS, such that serum NF-L improved model fit compared to WFNS

alone (pseudo R-squared values: WFNS: 0.225, serum NF-L: 0.128, WFNS + NF-L: 0.311, negative log-log link function).

Both CSF and serum NF-L independently predicted outcome when added to the Jaja core model predictors (Jaja *et al.*, 2018) (p=0.035 and p=0.037 for CSF and serum NF-L respectively), improving model fit (pseudo R-squared values: Jaja core model: 0.290, Jaja core model + CSF NF-L: 0.367, Jaja core model + serum NF-L: 0.348, negative log-log link function).

## Late NF-L rise in both CSF and serum predicts neurological outcome after SAH

In view of the prolonged rise in NF-L from day 4 onwards (Figure 1A), we next studied whether the area-under-the-curve (normalized per day) for NF-L from day 4 onwards was related to outcome, independent of WFNS. In order to be able to compare the relative impact of early and late NF-L levels on outcome and to avoid bias resulting from patients who had an indwelling ventricular catheter for longer than others and therefore provided late samples, this analysis was performed on the maximum number of patients with samples in both early (days 1-3) and late (days 4 onwards) time epochs: 23 patients had data points stretching between days 2 and 7.

Multivariable ordinal regression controlling for WFNS showed that NF-L level in the late epoch contributed to outcome additional to WFNS, whether it was assessed in CSF (odds ratio: 4.6, 95% CI: 1.6-13.4, p= 0.005) or serum (odds ratio: 2.4, 95% CI: 1.1-5.4, p= 0.03). However when both early and late CSF NF-L area-under-the-curve were analysed together, only late CSF NF-L retained significance (odds ratio: 3.9, 95% CI: 1.3-11.8, p= 0.017).

Late CSF and serum NF-L area-under-the-curve were higher in patients with radiological evidence of infarction, though this did not reach significance in an analysis of covariance controlling for age and WFNS (p=0.720 and p=0.184 for CSF and serum NF-L respectively, Supplementary Figure 2).

## Scavengeable haemoglobin is correlated with the brain injury marker NF-L

In order to substantiate the relationship of NF-L to pathological processes which are (1) specific to SAH, (2) delayed, and (3) potentially reversible, we selected extracellular haemoglobin as an example for two reasons. First, haemoglobin is specific to SAH. Second, it is released gradually and its CSF concentration increases with time during the first two weeks post-SAH, as clot lysis proceeds (Durnford *et al.*, 2015). Third, haemoglobin neurotoxicity

can be neutralized by intraventricular treatment with haptoglobin (Garland *et al.*, 2020), unless it undergoes oxidation (Faivre *et al.*, 1998) which prevents its binding to haptoglobin and renders it "unscavengeable". Establishing a relationship between NF-L and scavengeable haemoglobin would indicate that NF-L could be used as a surrogate biochemical marker of outcome in clinical trials of intrathecal haptoglobin treatment.

We quantified the fraction of scavengeable haemoglobin in the CSF after SAH (Figure 2A). In the first three days most CSF haemoglobin was bound by haptoglobin derived from the plasma accompanying the SAH (Figure 2B, Supplementary Figure 3A). From day 3 onwards, scavengeable haemoglobin predominated over non-scavengeable haemoglobin (Figure 2B; Supplementary Figure 3B-C); this increased to a maximum of 6.5μM (median, IQR 1.179-15.390) between days 11-13. From day 4 onwards, 83% of the haemoglobin present in the CSF following SAH was scavengeable, 10% was unscavengeable, and a small amount of haemoglobin, 7%, was already bound to endogenous haptoglobin (Figure 2B; Supplementary Figure 3).

To investigate the relationship between CSF scavengeable haemoglobin and NF-L levels we used multiple linear regression, controlling for WFNS, age and sex (Figure 3). With the maximum CSF NF-L value as the dependent variable, the highest preceding CSF scavengeable haemoglobin value was investigated as a predictor. CSF scavengeable haemoglobin level explained 50.3% of the variance in CSF NF-L level, with the p-value of the regression coefficient corresponding to the slope indicating a statistically significant dependence on scavengeable haemoglobin level ($p=0.0003$); neither age nor sex contributed significantly.

## NF-L exchange across the blood-brain interface differs from that of albumin

A circulating biomarker, such as serum NF-L, would be of immense value after SAH, since CSF would only be accessible in a minority of patients. Serum NF-L concentration would be expected to follow CSF NF-L due to brain-blood efflux, and indeed this was the case with the maximum CSF NF-L predicting the subsequent maximum serum NF-L over the sampling period (Figure 4A). The serum/CSF NF-L ratio represents the transfer of NF-L from CSF to blood. The serum/CSF NF-L ratio was lower in SAH versus controls (medians of 0.0057 vs 0.01 respectively, Mann-Whitney $p=0.024$), suggesting glymphatic efflux dysfunction after SAH.

NF-L (62kDa) has a similar molecular weight to albumin (60kDa), so we investigated whether NF-L efflux and albumin influx across the blood-brain interface, albeit in different

directions, were synchronized, since this may provide insight into the routes of exchange of these two molecules. Albumin influx from the blood into the CSF occurs via the choroid plexus and the blood-brain barrier (BBB) (Thompson, 2005). Since albumin is not synthesized in the CNS, the ratio between CSF and serum albumin concentration – the albumin quotient, or Qalb, is used as a proxy for BBB permeability (Tibbling *et al.*, 1977). An increase in this ratio indicates that there is increased BBB permeability to molecules of similar size to albumin.

There is evidence that BBB permeability increases after SAH (Galea *et al.*, 2012). To investigate changes in BBB permeability, paired CSF and serum samples from SAH patients taken at day four or later were assayed for albumin. Time points earlier than day four were excluded since analysis at these time points would have been confounded by albumin that had entered the CSF with the bleed (Supplementary Figure 4). Figure 4B shows there is a sustained elevation of Qalb after SAH from day 4 onwards relative to the control population (red line). An unpaired t-test between the control population and the maximum Qalb in the SAH patients shows a highly significant difference (Figure 4C).

NF-L may exit the brain into the circulation via a number of routes, including CSF drainage via the cribriform plate, nerve roots and dural lymphatics, the intramural peri-arterial pathway and reflux across the gliovascular membrane complex at the BBB (Rasmussen *et al.*, 2018). If the BBB, namely the gliovascular membrane complex at capillary level, was the route for NF-L efflux, one would expect a positive correlation between the serum/CSF NF-L ratio and Qalb. Hence we explored the relationship between the mean serum/CSF NF-L ratio and Qalb. This showed a significant negative correlation (Figure 4D). This finding was robust to sensitivity analyses, which included using the maximum Qalb and the serum/CSF NF-L ratio on the same day, and adding CSF drainage volume as a covariate. In summary, NF-L exchange across the blood-brain interface differs from that of albumin. This phenomenon is not specific for SAH, since we reproduced the same observation in an independent set of samples from clinical neurochemistry practice, representing a wide range of serum/CSF NF-L ratios and Qalb results (Supplementary Figure 5).

## Discussion

SAH results in a long term deficit in neurological function, yet outcome prediction is challenging. Here we take the novel approach of combining WFNS as a clinical marker of early brain injury, and NF-L as an independent biomarker integrating multiple post-injury

processes, to best model long-term neurological outcome in SAH patients. An early CSF or serum NF-L estimation, within the first 1-3 days after SAH, adds value to the WFNS, in the prediction of six month outcome after SAH, explaining an additional circa 10% in variance on top of the WFNS. A serum biomarker is significantly advantageous over a CSF biomarker, since CSF may not be readily available in all patients. EVD placement is required for clinical management in only a third of patients (Galea *et al.*, 2017), and lumbar CSF drainage is not a mainstay of treatment.

This study extends and clarifies research examining neurofilament subunits, namely heavy (Petzold *et al.*, 2005; Petzold *et al.*, 2006; Lewis *et al.*, 2008) and light (Nylen *et al.*, 2006; Zanier *et al.*, 2011; Halawa *et al.*, 2018; Hviid *et al.*, 2020) chains, following SAH. These studies are summarized in Supplementary Table 2. NF-L is a cytoskeletal protein widely expressed in neurons which is emerging as a robust marker of brain injury (Gaetani *et al.*, 2019). Pre-symptomatic disease, such as mild cognitive impairment (Kern *et al.*, 2019), may be theoretically possible in some control individuals. Hence in the idealistic scenario where all control individuals are guaranteed to be free of subclinical neurodegenerative disease, the difference between SAH and controls would be even more accentuated.

The early prognostic effect of NF-L suggests that its release is likely to be linked to early brain injury. The latter triggers a number of prolonged processes such as inflammation and oxidative stress while other processes have a delayed onset such as delayed cerebral ischemia, haemoglobin toxicity, ferro-toxicity and other as yet unidentified mechanisms. Hence it is not surprising that CSF NF-L area-under-the-curve from day 4 onwards had a bigger impact on six month outcome, compared to CSF NFL area-under-the-curve on days 1-3. While it is not clinically practical to measure NF-L levels repeatedly, area-under-the-curve analysis here provided biological insight. In particular, the dramatic contribution of CSF NF-L area-under the curve in the late epoch suggested that significant neuronal damage occurs in a delayed manner, and this impacts significantly on outcome. It is very possible that early brain injury directly following SAH may interact with secondary processes to determine outcome.

One of the prolonged secondary pathological processes likely to be integrated in the NF-L rise after SAH, is the gradual release of haemoglobin from the clot. Extracellular haemoglobin is directly neurotoxic (Garland *et al.*, 2020) and also triggers tertiary processes such as vasoconstriction, inflammation and iron deposition (Bulters *et al.*, 2018), which themselves propagate neuronal damage. Intracranial haptoglobin treatment has potential to prevent these effects (Garland *et al.*, 2020). In order to determine whether NF-L could be used

as a surrogate marker for treatment trials of haptoglobin, we considered the proportion of haemoglobin able to bind haptoglobin. This "scavengeable" haemoglobin makes up most of the CSF haemoglobin after day 3 post-SAH. Here we show that peak CSF NF-L was strongly associated with preceding peak CSF "scavengeable" haemoglobin, suggesting that CSF NF-L could be useful as a surrogate marker for efficacy of intracranial haptoglobin in Phase II trials. It also demonstrates a link between NF-L and a delayed pathological process such as haemoglobin toxicity.

Not surprisingly, the clinical significance of raised serum NF-L followed that of CSF NF-L. A number of factors are likely to affect the relationship between CSF and serum NF-L, as NF-L generated intrathecally is transferred to the circulation, including blood-brain transfer rate and circulating half-life. Nevertheless serum NF-L correlated with CSF NF-L, in keeping with the prognostic value of NF-L in both compartments.

The BBB was compromised after SAH, as confirmed by the high Qalb in SAH patients compared to controls; this has been previously reported (Galea *et al.*, 2012). After experimental SAH, damage to the capillary basement membrane (Scholler *et al.*, 2007), increased pinocytosis and opening of tight junctions (Doczi, 1985) causes BBB breakdown.

The serum/CSF NFL ratio can be considered to be a marker of the rate of solute drainage from the brain. This ratio was decreased in SAH patients versus controls, in keeping with glymphatic efflux dysfunction, which has been recently demonstrated in an animal model of SAH (Pu *et al.*, 2019). Surprisingly, brain-blood NF-L transfer correlated negatively with blood-brain albumin transfer. This novel observation was not specific to SAH, since we observed a similar pattern in an unselected population of neurological patients. Impaired glymphatic clearance in those with a damaged BBB is a likely explanation – the co-occurrence of glymphatic dysfunction and BBB disruption in brain pathology has been previously discussed and postulated (Verheggen *et al.*, 2018), and our observation is in keeping with this idea. It appears that NF-L and albumin use different routes of transfer across the blood-brain interface. Hence NF-L transfer from brain to blood does not occur across a leaky BBB in the capillary bed, but rather other pathways such as CSF efflux across the cribriform plate, nerve roots sleeves and dural lymphatics, and interstitial fluid efflux pathways across paravascular routes (Rasmussen *et al.*, 2018).

The highlight of this work is the prognostic usefulness of CSF and serum NF-L in predicting clinical outcome after SAH. Further work is required to progress these findings in larger more generalizable cohorts, including SAH patients with lower Fisher grades. We introduce the novel concept of including a biomarker integrating an array of early and

secondary processes, in predictive modelling of SAH outcome. NF-L is used here as an example; other radiological or biochemical markers may be better, or their addition may improve prediction. The use of such variables has potential to transform modelling of long-term clinical outcome after SAH.

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## Competing interests

JM is a current employee of Bio Products Laboratory Limited, a plasma-derived therapeutics manufacturing company. HZ has served at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (all outside submitted work).

## Author notes

PG and MJM contributed equally. HZ, DB and IG are joint senior authors. IG, DB, HK and PG designed different aspects of the study; PG, MJM JT, and AJH performed experiments; HK and IG supervised experiments; BG, AZ and AD collected human samples and clinical data; DB and IG supervised the clinical part of the study; PG, MJM, HK and IG analysed data; PG and IG wrote the first draft of the manuscript; all authors revised the manuscript.

## Figures legends

**Figure 1. CSF and serum NF-L predict long-term neurological outcome after SAH.** (**A**) Serial CSF and serum NF-L over a two week period after SAH (median ± inter-quartile range (IQR, shaded zone), n numbers under x axis). (**B**) In both CSF and serum, the maximum NF-L observed for each patient over the two week sampling period was significantly greater than the control population (mean ± 95% CI; unpaired t-test, p<0.0001. CSF: control=19, SAH=42. Serum: control=17, SAH=41). (**C**) Ordinal logistical regression shows high CSF NF-L between days 1-3 predicts poor outcome at 6 months on the modified Rankin Scale (odds ratio 4.3, 95% CI 1.1-16.2; pseudo $R^2$ = 0.093, n= 24 and 12 for high and normal CSF NF-L respectively). (**D**) Ordinal logistic regression shows high serum NF-L predicts poor outcome at 6 months on the modified Rankin Scale (odds ratio 2.3, 95% CI 0.6-6.6; pseudo $R^2$ = 0.086, n= 7 and 27 for high and normal serum NF-L respectively). High CSF and serum NF-L were defined as higher than the upper limit of the respective control population-based reference ranges. The NF-L reference range was defined as the mean plus and minus 1.96 standard deviations of the control population log-normal distribution.

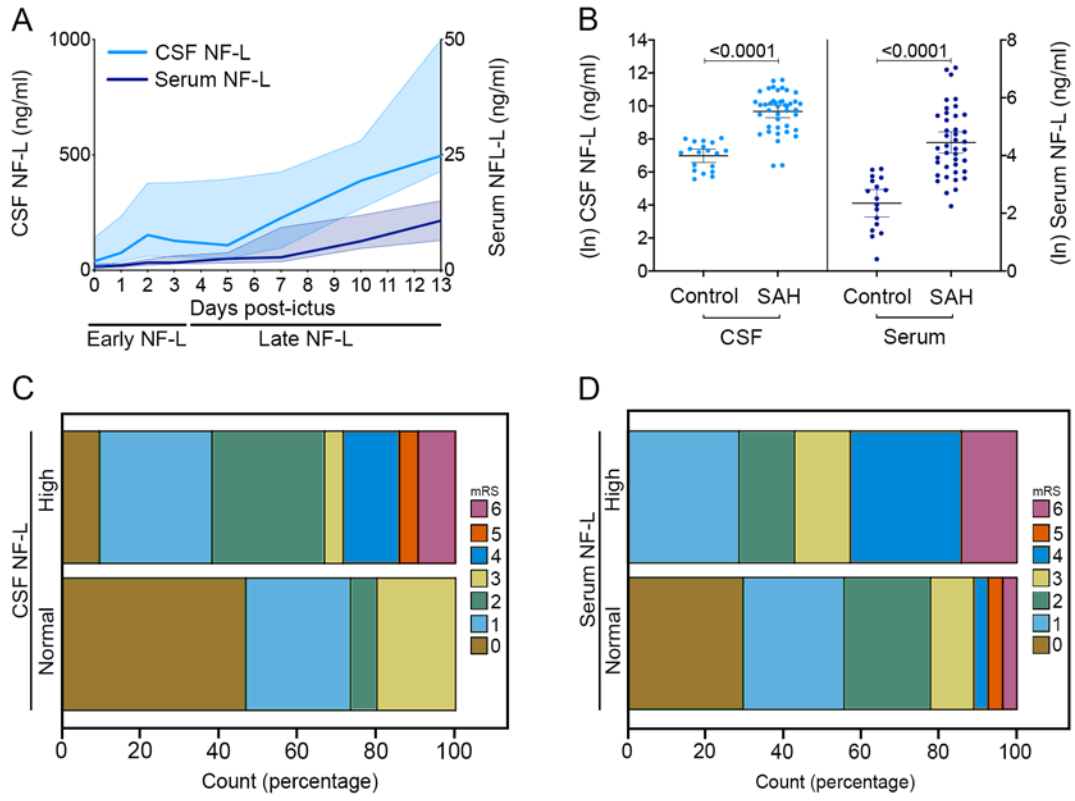


**Figure 2. Scavengeable haemoglobin is elevated in the CSF of SAH patients.** (**A**) size-exclusion chromatography to identify haemoglobin species. Neat or Hp-saturated samples reveal haem containing haemoglobin peaks in the 415nm Soret band. (**B**) pie charts, representing the relative proportions of bound, scavengeable and unscavengeable haemoglobin over the two-week sampling period. Increasing diameter reflects increasing total haemoglobin. Days and day groupings indicated in figure.

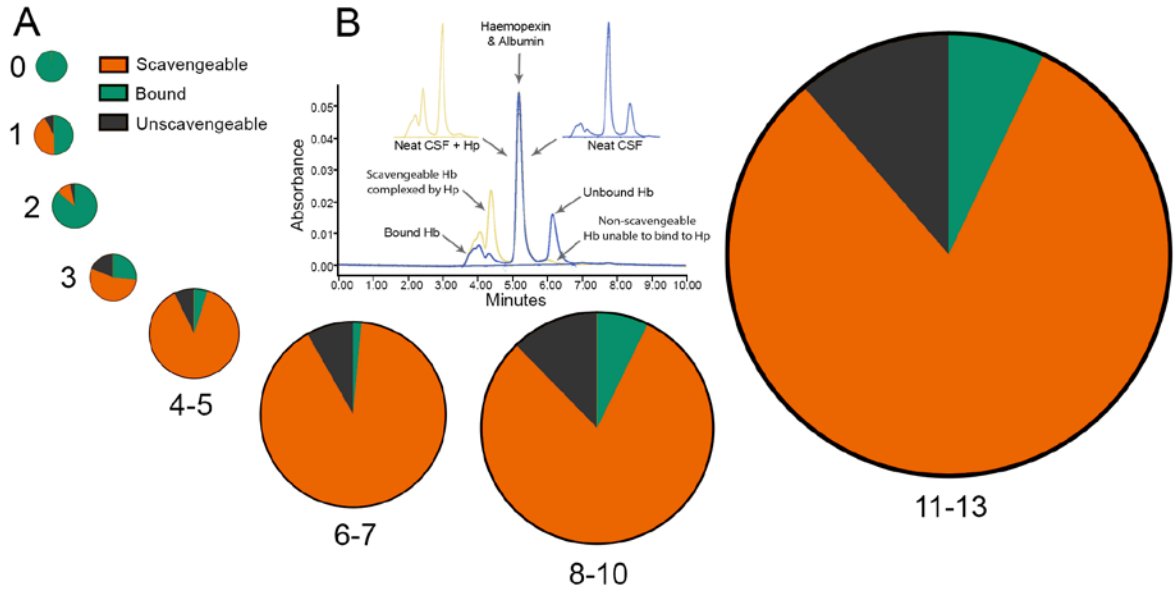


**Figure 3. Scavengeable haemoglobin is correlated with the brain injury marker NF-L.** Multiple linear regression was used to predict CSF NF-L levels using the highest preceding scavengeable haemoglobin, while controlling for age and sex ($R^2$ = 0.503, scavengeable haemoglobin p=0.0003; n=36); scavengeable Hb plotted against model fitted (i.e., predicted) CSF NFL values.

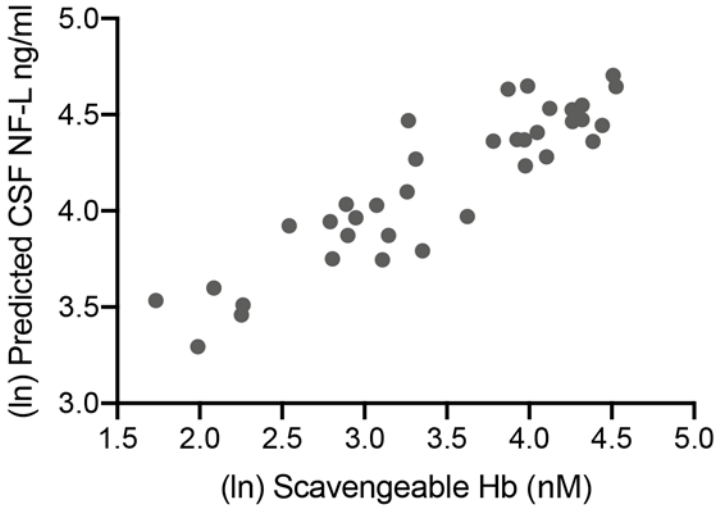
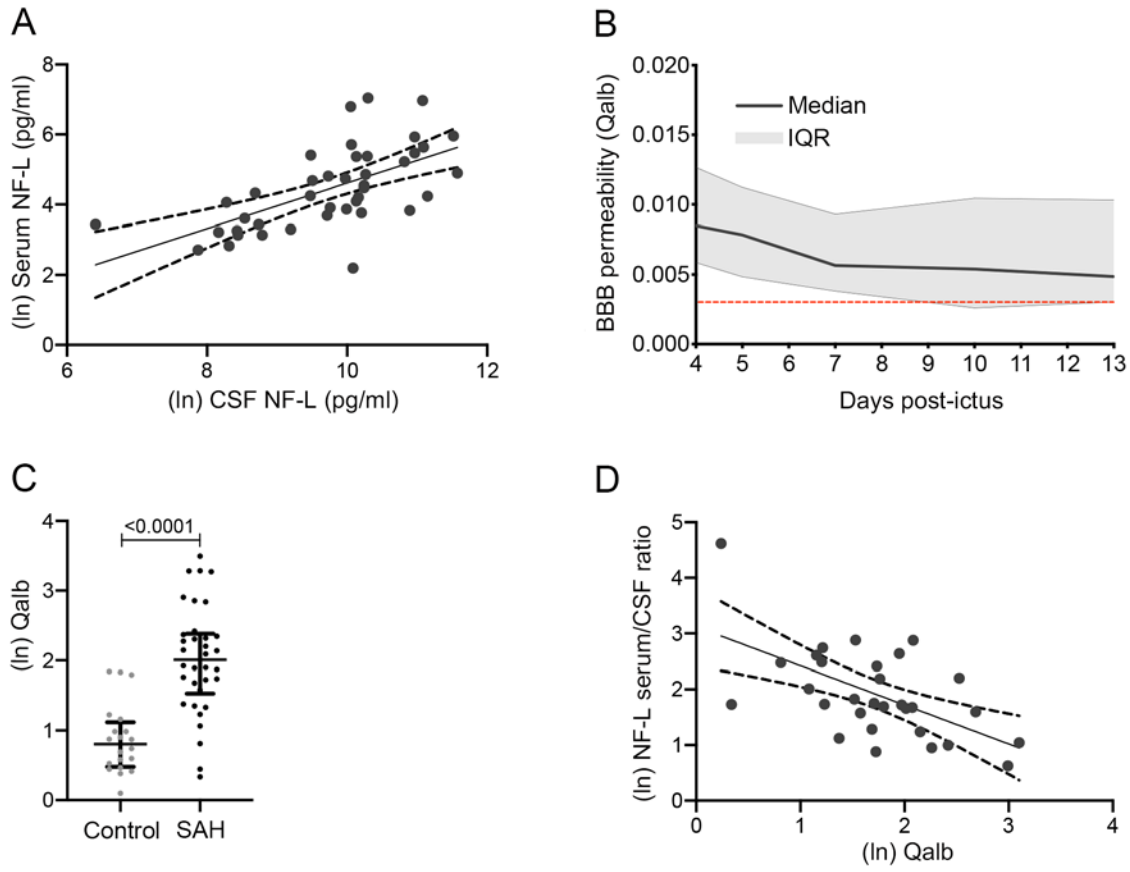


**Figure 4. Brain-to-blood NF-L efflux.** (**A**) Scatterplot of CSF NFL vs serum NFL reveals a significant Pearson r correlation (r=0.62, p<0.0001, n=41). (**B**) Qalb is elevated from day 4 until the end of the two-week sampling period. The red line is the mean value for the control population. (**C**) the maximum Qalb for each patient over the two-week sampling period is significantly greater than the control population (mean ± s.e.m; unpaired t-test, p<0.0001, n= 20 and 34 for control and SAH respectively). (**D**) Scatterplot of Qalb vs the serum/CSF NFL ratio reveals a significant negative Pearson r correlation (r=-0.58, p=0.0008, n=42).



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**SUPPLEMENTARY MATERAL FOR:**

# Neurofilament light predicts neurological outcome after subarachnoid haemorrhage

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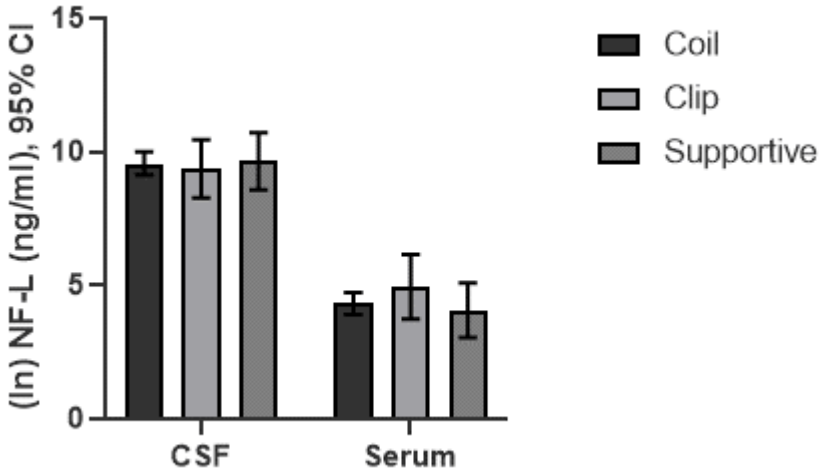
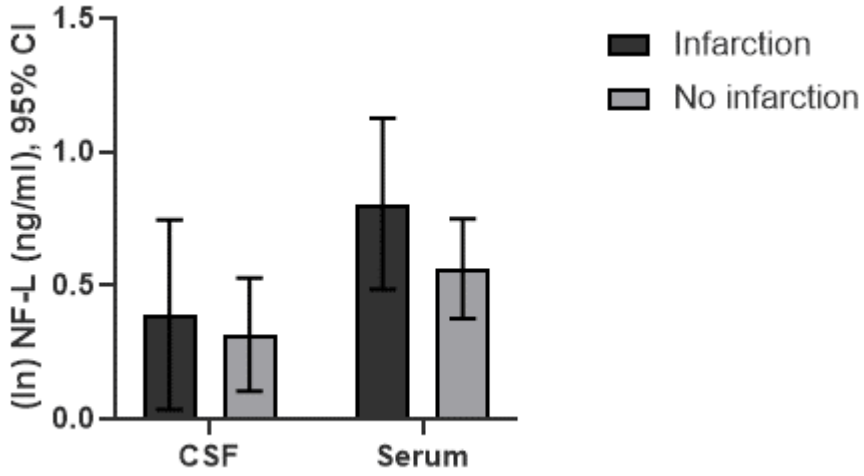
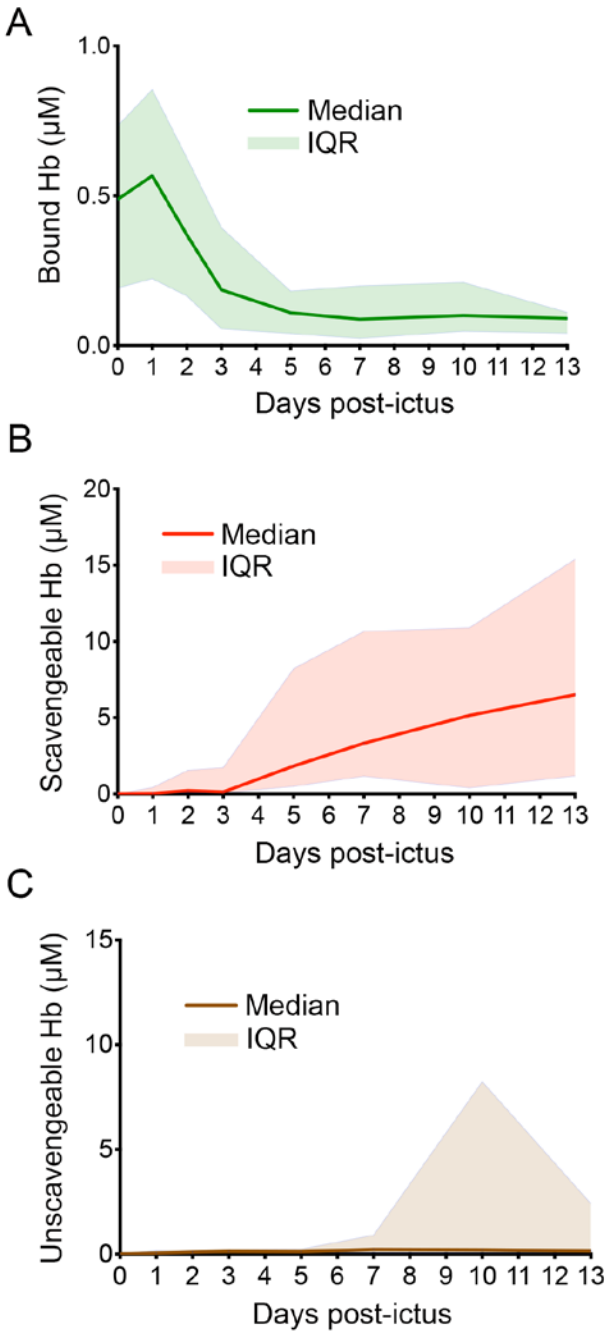
**Supplementary Figure 1. CSF and serum NF-L were not significantly different between patients who had surgery, coiling, or supportive management.** Plot shows estimated marginal means (with 95% confidence intervals) for maximum CSF and serum NF-L from analyses of covariance controlling for age and WFNS ($F(2,37)=0.077$, $p=0.926$ for CSF NF-L and $F(2,36)=0.685$, $p=0.511$ for serum NF-L).



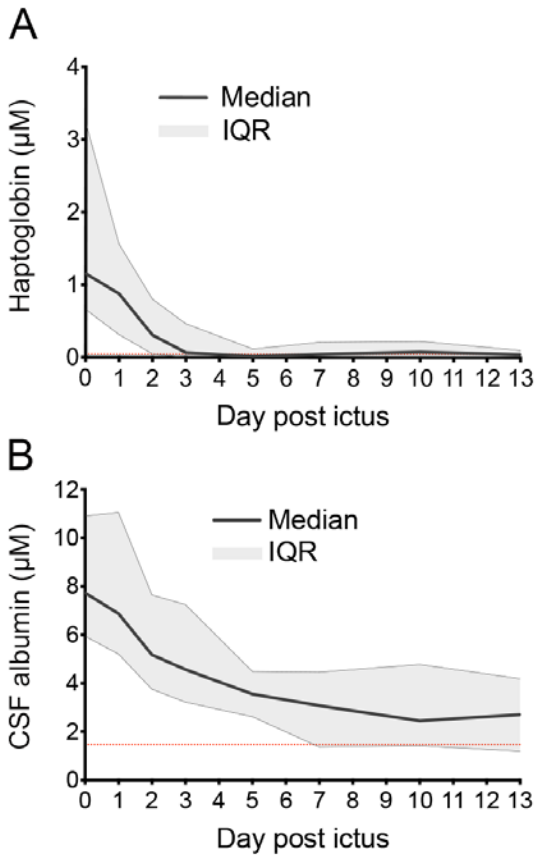
**Supplementary Figure 2. Late CSF and serum NF-L area-under-the-curve estimated marginal means, adjusted for age and WFNS, in patients with radiological evidence of infarction.** Plot shows estimated marginal means (with 95% confidence intervals) for late CSF and serum NF-L area-under-the-curve (normalized per day) from an analysis of covariance controlling for age and WFNS ($F(1,28)= 0.131$, $p=0.720$ for CSF NF-L and $F(1,30)= 1.849$, $p=0.184$ for serum NF-L).



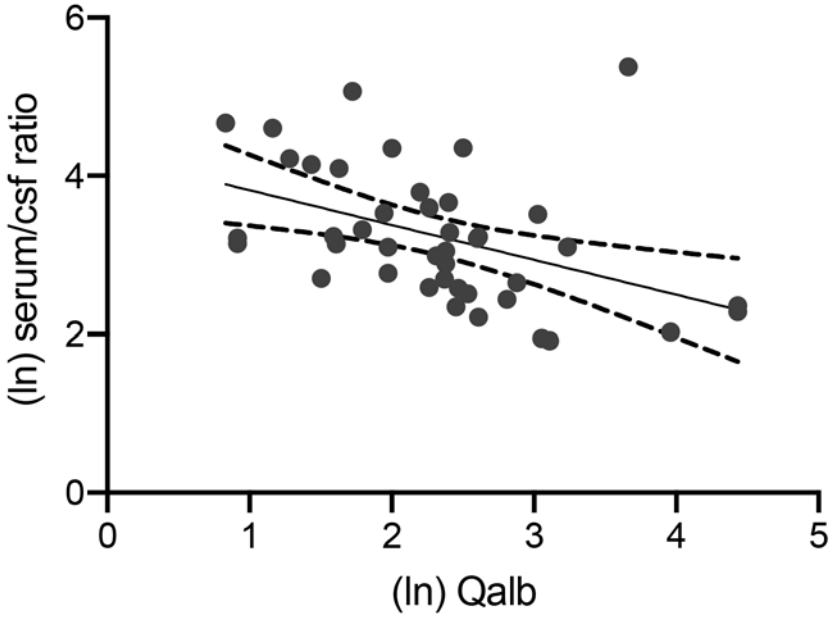
**Supplementary Figure 3. UPLC characterisation of haemoglobin species accumulation following SAH.** (**A**) Haemoglobin that is bound to endogenous Hp, 'Bound haemoglobin', plateaus after 3 days post-ictus. (**B**) Haemoglobin that can bind haptoglobin, 'scavengeable haemoglobin', gradually increases over the two-week sampling period. (**C**) Haemoglobin that cannot bind Hp, 'unscavengeable haemoglobin', is at minor amounts over the two-week sampling period. All data median ± interquartile range.



**Supplementary Figure 4. Plasma proteins haptoglobin and albumin are present at a high concentration in the CSF in the first three days after SAH.**



**Supplementary Figure 5. Serum/CSF NF-L ratio correlates negatively with Qalb.** Performed in an independent set of unselected samples from a clinical neurochemistry laboratory, representing a wide range of serum/CSF NF-L ratios and Qalb results (p=0.0035, r=-0.44).



**Supplementary Table 1. Clinical characteristics of included patients and controls**

| | Non-traumatic SAH patients | Control patients | p |
|---|---|---|---|
| Number | 42 | 19 | |
| Age (years)[a] | 63 (21-82) | 67 (21-92) | 0.80[g] |
| Gender[b] | | | |
| Male | 15 (35.7%) | 5 (26.3%) | 0.56[h] |
| Female | 27 (64.3%) | 14 (73.7%) | |
| Premorbid hypertension[b] | | | |
| Yes | 18 (42.9%) | | |
| No | 24 (57.1%) | | |
| Initial WFNS[b] | | | |
| 1 | 4 (9.5%) | | |
| 2 | 8 (19%) | | |
| 3 | 3 (7.1%) | | |
| 4 | 20 (47.6%) | | |
| 5 | 7 (16.7%) | | |
| Fisher grade[b] | | | |
| 3 | 2 (4.8%) | | |
| 4 | 40 (95.2%) | | |
| Delayed ischaemic neurological deficit[c] | | | |
| Yes | 5 (12.2%) | | |
| No | 36 (87.8%) | | |
| Radiological evidence of ischaemia[c] | | | |
| Yes | 10 (24.4%) | | |
| No | 31 (75.6%) | | |
| Aneurysmal management[b] | | | |
| Coiled | 32 (76.2%) | | |
| Clipped | 5 (11.9%) | | |
| Supportive | 5 (11.9%) | | |
| Aneurysm location[b,d] | | | |
| Anterior cerebral artery[e] | 16 (38.1%) | | |
| Middle cerebral artery | 5 (11.9%) | | |
| Posterior circulation[f] | 14 (33.3%) | | |
| Internal carotid artery | 3 (7.1%) | | |
| No vascular abnormality | 4 (9.5%) | | |

a, median and range
b, number and %
c, information available for 41 patients
d, the ruptured aneurysm is indicated in cases with multiple aneurysms
e, included anterior cerebral, anterior communicating and pericallosal arteries
f, included posterior cerebral, posterior communicating, posterior inferior cerebellar and basilar arteries
g, Mann-Whitney test
h, Fisher's exact test

## Supplementary Table 2. Summary of main characteristics of previous studies examining neurofilament subunits after SAH

| | | | Garland et al | Petzold et al 2005 | Petzold et al 2006 | Nylen et al 2006 | Lewis et al 2008 | Zanier et al 2010 | Halawa et al 2017 | Vinter Bodker Hviid et al 2020 |
|---|---|---|---|---|---|---|---|---|---|---|
| **PMID** | | | current study | 15785235 | 16705199 | 16806706 | 18319731 | 20571038 | 29164612 | 31808039 |
| **SAH patients (n)** | ≥40 | <40 | 42 | 10 | 17 | 48 | 30 | 35 | 19 | 44 |
| **Controls within study (n)** | ≥10 | None | 19 | 20 | None | None | None | 13 | None | 44 |
| **Controls: neurodegeneration / inflammation excluded?** | Yes | No or N/A | Yes | No | N/A | N/A | N/A | Yes | N/A | Yes |
| **Biomarker** | | | NF-L | NF-H | NF-H | NF-L | NF-H | NF-L | NF-L | NF-L |
| **Source of CSF in SAH patients** | Done | Not done | EVD | EVD | EVD | LP | EVD | EVD | EVD | Not done |
| **Source of CSF in controls** | Done | Not done | Lumbar | Lumbar | Not done | Not done | Not done | Lumbar | Not done | Not done |
| **Paired CSF/serum in patients** | Yes | No | Yes | No | No | No | Yes | No | No | No |
| **Paired CSF/serum in controls** | Yes | No | Yes | No | No | No | No | No | No | No |
| **Sampling period (days post-ictus)** | Included days 1-3 | After day 3 / Not defined | days 1-14 | days 1-8 | days 1-14 | day 11 only | days 1-12 | Not defined | days 4 and 10 | 4 hours and day 1 |
| **CSF assay** | Done | Not done | ELISA | ELISA | ELISA | ELISA | ELISA | ELISA | ELISA | Not done |
| **Serum assay** | Done | Not done | SIMOA | Not done | Not done | Not done | ELISA | Not done | Not done | SIMOA |
| **Primary clinical outcome measure** | Studied | Not studied | 6 months mRS | Not studied | 3 month GOS | 12 month GOSE | 6 months GOSE | 6 month GOS | DCI | day 30 mRS |
| **Association of primary outcome with CSF NF** | + | Not studied / detected | + | Not studied | + | + | Not studied | Not detected | Not detected | Not studied |
| **Association of primary outcome with serum NF** | + | Not studied /detected | + | Not studied | Not studied | Not studied | + | Not studied | Not studied | + |
| **Multivariable regression with WFNS** | Done | Not studied | Done | Not studied | Not studied | Not studied | Not studied | Not studied | Not studied | Done |
| **Outcome prediction: early versus late comparison** | Done | Not studied | Done | Not studied | Not studied | Not studied | Not studied | Not studied | Not studied | Not studied |
| **Mechanistic studies** | Done | Not studied | Done | Done | Not studied | Not studied | Done | Not studied | Not studied | Not studied |