

Supplementary Methods for:

Haptoglobin genotype and outcome after aneurysmal subarachnoid haemorrhage

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Subjects and Methods

GOSH study

Clinical data and DNA was collected from patients with aSAH enrolled in the GOSH study, designed to examine the genetic and clinical characteristics of patients with ruptured and unruptured intracranial aneurysms. The GOSH study recruited at 22 tertiary neurosurgical centres in the UK between 2011 and 2014. Written informed consent was obtained from participants, or next of kin if patients lacked capacity. Recruitment was from both inpatient and outpatient neurovascular clinics following either a new or previous diagnosis respectively; patients who died early after aSAH were not recruited. Standardized case report forms were completed by trained stroke research practitioners. The study was approved by the National Research Ethics Committee (NRES reference no: 09/H0716/54).

Outcomes, covariates & definitions

The primary outcome measure was the modified Rankin scale (mRS) at follow up, dichotomized into favourable (mRS 0-1) and unfavourable (mRS 2-6) outcomes, administered by qualified research practitioners at the time of assessment. The choice of this instrument and dichotomization threshold was based on data availability in this population of aSAH survivors. The modified version¹ of the Rankin Scale² was used throughout in a standardized way, ranging from 0 (no symptoms at all) to 5 (severe disability); mRS 6 (death) was added to include mortality³.

Covariates included age, sex, admission WFNS score⁴, admission Fisher grade⁵, hydrocephalus, aneurysmal treatment (coiling, clipping, or none), time since ictus, centre, smoking pack years, presence or absence of nimodipine treatment, diabetes mellitus, hypercholesterolaemia, hypertension, anti-hypertensive medication, and non-SAH related disability affecting the primary outcome measure. We defined hypertension, hypercholesterolaemia and diabetes mellitus as present if the patient or medical records indicated the condition for which either drug treatment, lifestyle, or other advice had been provided.

Control population

A sample of 927 individuals from the ALSPAC cohort^{6,7}, previously genotyped for the *HP* CNV (see below), was used as the control population. Plasma haptoglobin level was available for 325 of these individuals. It was measured using an immunoturbimetric haptoglobin assay (Cobas Integra kit catalogue number 03005593 322, Roche, USA) on a Hitachi Cobas c311 autoanalyser. In the ALSPAC study, pregnant women resident in Avon, UK with expected dates of delivery 1st April 1991 to 31st December 1992 were invited to take part in the study. Of the 15,247 pregnancies, there

were 14,899 children who were alive at 1 year of age. The ALSPAC study website (<http://www.bristol.ac.uk/alspac/researchers/our-data/>) contains details of all the data that is available through a fully searchable data dictionary and variable search tool. Ethical approval for the study was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees.

GOSH HP CNV genotyping

We performed *HP* CNV genotyping using a quantitative polymerase chain reaction (qPCR) method⁸. The assay amplified a region in the 5' terminal of the *HP* gene's first exon as an internal control (HP5'), and the breakpoint of the *HP* duplication (HP2). The HP2/HP5' ratio (theoretically either 0, 1, or 2) was used to determine the genotype as HP1-1, HP2-1 or HP2-2 respectively. Samples were run in triplicate, and triplicates with a HP2/HP5' ratio coefficient of variation >10% were re-assayed. A second method of *HP* genotyping by PCR⁹ was performed on samples with HP2/HP5' ratio values between 0.4619 and 0.6214, in order to confirm the *HP* CNV genotype. For quality control, we randomly selected 10% of samples not previously subject to this typing method and genotyped them via this method, comparing results to the qPCR technique. 98% concordance was observed; we repeated discordant samples using both assays and found them to be in agreement. There was only one failed genotype call, i.e. a 99.92 % call rate.

GOSH rs2000999 genotyping

rs2000999 has been shown to be associated with haptoglobin levels in the plasma and its level of expression in tissue, as exemplified by adipose tissue¹⁰. Since rs2000999 is downstream of the *HP* gene, its effect is probably mediated via linkage disequilibrium with upstream variations, such as rs35283911¹¹. We genotyped patients for rs2000999 status using Kompetitive Allele Specific PCR (KASP), a fluorescence resonant energy transfer (FRET) PCR based assay (LGC Genomics Limited, Hertfordshire, UK). Genotypes were called automatically by SNPviewer v4.0 (LGC Genomics Ltd., Hertfordshire, UK). We genotyped cases marked equivocal by the software (n=51) using a Taqman assay (C_11439054_10, ThermoFisher, USA). Cluster plots were viewed using Taqman genotyping software (v1.4, Applied Biosystems, USA) to call genotypes. For quality control, 30 other cases typed by the KASP assay and successfully called by SNPviewer were cross-checked with the Taqman assay and 100% concordance was observed. Nine out of 1299 samples failed to be called by both KASP and Taqman methods, resulting in a 99.31% call rate.

ALSPAC HP CNV genotyping

The HP CNV of ALSPAC children was typed using amplification ratio control system (ARCS), a validated liquid phase high-throughput assay for quantifying gene copy number ¹². Out of 1056 samples, 927 were successfully called (ie 87.8%).

ALSPAC rs2000999 genotyping

ALSPAC children were genotyped using the Illumina HumanHap550 quad chip genotyping platforms by 23andme subcontracting the Wellcome Trust Sanger Institute, Cambridge, UK and the Laboratory Corporation of America, Burlington, NC, US. The resulting raw genome-wide data were subjected to standard quality control methods. Individuals were excluded on the basis of sex mismatches; minimal or excessive heterozygosity; disproportionate levels of individual missingness (>3%) and insufficient sample replication (IBD < 0.8). Population stratification was assessed by multidimensional scaling analysis and compared with Hapmap II (release 22) European descent (CEU), Han Chinese, Japanese and Yoruba reference populations; all individuals with non-European ancestry were removed. rs2000999 genotype was extracted from this dataset.

Biochemistry – high Fisher grade aSAH

44 Fisher grade III-IV aSAH patients were recruited at the Southampton centre, after approval by the National Research Ethics Committee (reference no: 12/SC/0666). CSF was obtained from external ventricular drains (EVD) on alternate days from insertion and up to two weeks or until the EVD was removed. CSF was spun and frozen within one hour of sampling. We did not use CSF samples in the event of an EVD infection.

We performed haemoglobin-haptoglobin complex quantification, irrespective of oxidation state, using size exclusion ultra-performance liquid chromatography (UPLC) with absorbance measurement at 415nm. A 9 point Hb standard curve (0 to 1 mg/ml) was prepared from commercially-available lyophilized human Hb (Sigma) 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 quantification at 570 nm using a HemocueTM (Hemocue, Sweden). We determined accuracy of the standard curve to be 3.3% using a Hb control. 50µL of neat CSF was loaded onto the UPLC column using a running buffer consisting of 50 mM Tris and 150 mM NaCl, at pH 7.5. Bound and free Hb peaks' area under the curve was quantified against the Hb standard curve. We quality controlled each assay run using three haemoglobin-haptoglobin complex standards (200 µg/ml, 10 µg/ml and 1 µg/ml) covering the dynamic range of the assay. We determined haptoglobin phenotype using two methods: inspection of serum UPLC chromatograms ¹³ and non-denaturing Western blot using 1:5000 polyclonal rabbit anti-haptoglobin antibody (Sigma, Gillingham, Dorset, UK), with 100% concordance.

CSF/serum albumin ratio (Qalb) was determined after measurement of albumin in serum and CSF by rate nephelometry on an IMMAGE Immunochemistry system (Beckman Coulter). Qalb was only measured on day 4 post-ictus onwards, to ensure reliability as a measure of blood-brain barrier permeability, since preliminary data (not shown) established that three days were required for plasma proteins derived from the bleed to be cleared from the intrathecal compartment. For this reason, Qalb was only available in 19 aSAH patients.

Biochemistry – low Fisher grade aSAH

CSF samples from 8 patients with aSAH Grade I-II were identified retrospectively during an ongoing service evaluation of lumbar puncture at the Southampton centre. We excluded cases with delayed presentation (>10 days) and traumatic/repeat lumbar punctures. Xanthochromia was assessed on a UVIKON XS spectrophotometer using Bio-C software (NorthStar Scientific, Bedfordshire, UK). We determined Hb concentration using the Beer-Lambert equation, using the net Hb absorbance at 415nm and an extinction coefficient of 141.2¹⁴.

Clot volume

Computed tomographic (CT) imaging of the head was available for 38 out of the 44 patients with high-grade aSAH providing CSF; there was no significant difference in the demographics and baseline characteristics of these 38 patients compared to the whole cohort (data not shown). Volumetric blood clot volume was quantified using MIPAV (Medical Image Processing, Imaging and Visualization) v7.2. We only included CT images in the analysis if acquired using the same imaging protocol within the first 3 days post-SAH, using contiguous slices. Image radiodensity threshold was set between 50 and 80 Hounsfield units, and converted to a binary mask. We manually drew regions of interest representing subarachnoid and total blood clot on each slice, and grouped them into single three-dimensional volumes.

Statistics

To evaluate the association between plasma haptoglobin level and *HP* CNV, we performed separate multivariable linear regression modelling in R using the ALSPAC cohort (n=325). We considered *HP* CNV as the exposure and plasma haptoglobin level as the outcome, adjusting for rs2000999 and sex.

For GOSH, we conducted multivariable logistic regression modelling in SPSS v23 and R. A logistic regression model was constructed, with mRS as binary dependent variable, and *HP* CNV, rs2000999 and other covariates listed in Table 1 as independent variables. We dichotomized Fisher grade into low (I-II) and high (III-IV) as per consensus in aSAH studies. We tested interactions

between the *HP CNV* / rs2000999 and other covariates for model fit. Only the *HP CNV* x Fisher category interaction improved model fit ($p < 0.001$). We performed sensitivity analysis with progressively decreasing length of follow-up, GOS as outcome, dichotomization of mRS into favourable (mRS 0-2) and unfavourable (mRS 3-6) outcomes, and non-dichotomized Fisher grade. Multiple imputation was performed using the iterative Markov Chain Monte Carlo method, with 10 iterations and 2×10^6 as the initialization value for the Mersenne twister random number generator. All variables were used and WFNS was dichotomized. The imputation was repeated for a total of five times; imputed values were very similar between the five imputations. Dichotomized mRS was regressed on the same variables as in the complete case analysis. One centre with one patient, which was missing from the complete case analysis, was also excluded from the imputed case analysis, since the quasi-complete separation in data for this centre prevented the logistic regression model from converging.

In the biochemical study, all scalar variables were non-parametric except age, so they were either natural log-transformed prior to ANOVA/ANCOVA or analysed with non-parametric tests. We performed analysis of covariance in SPSS with maximum ln haemoglobin-haptoglobin complex as the dependent variable, ln clot volume, age and maximum ln Qalb as scalar covariates and *HP CNV* genotype and sex as fixed factors. Analyses using mean values for haemoglobin-haptoglobin complex and Qalb showed similar results. For all studies, two-tailed hypotheses were tested with $\alpha = 0.05$.

Table 1. Biospecimen protocol and methodology reporting recommendations

From: Chou SH, Macdonald RL, Keller E; Unruptured Intracranial Aneurysms and SAH CDE Project Investigators. Biospecimens and Molecular and Cellular Biomarkers in Aneurysmal Subarachnoid Hemorrhage Studies: Common Data Elements and Standard Reporting Recommendations. *Neurocrit Care.* 2019 Jun;30(Suppl 1):46-59. doi: 10.1007/s12028-019-00725-4.

Core data element recommendations	
Biological tissue sample source	CSF
Conditions included/excluded	Only aneurysmal SAH was included. Other forms of SAH were excluded.
Baseline/time-zero specimen	Not applicable; all samples were collected after ictus.
Site and method of sample acquisition	CSF was ventricular. CSF was drawn from a three way tap connecting the ventricular catheter (approximately 30cm long) to the tubing leading to an external CSF drainage and monitoring system (Becker®, Medtronic). For sampling, the tap was opened to the ventricular catheter, and closed to the drainage system. The first 3ml of CSF (representing dead space) was discarded to ensure fresh CSF was obtained.
Timing of biospecimen collection	Alternate days from insertion of external ventricular drain (EVD) up to two weeks or until the EVD was removed
Type of collection tube	Sterile polystyrene
Method of biospecimen processing	<ul style="list-style-type: none"> • Centrifugation to separate supernatant from cellular debris and separate storage was performed. • Parameters: 10 min, soft brakes, 20°C, 1500 rcf.
Time lapse between sample collection and processing	60 minutes maximum
Method of biospecimen storage	<ul style="list-style-type: none"> • Storage at – 80°C in cryotubes • No freeze/thaw cycles

Supplemental data element recommendations	
Control biospecimens	Not applicable
Convalescent biospecimens	Not applicable
Serial biospecimen collection	For serial collection, consistent method of acquisition including site of acquisition was

	used, to minimize variance in biospecimen and biomarker analyses
Biospecimen storage	Storage was at -80°C in 100/450µL aliquots labelled with printed information (patient ID, time post-ictus, sample identity (CSF), sample date, volume) and an Excel-based inventory system
Biomarker analysis	No freeze/thaw cycles
Selective inhibitors use	Not used
Biospecimen transport and shipping	Samples transferred on dry ice

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