Haptoglobin genotype and outcome after aneurysmal subarachnoid haemorrhage

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Abstract

Objective: After aneurysmal subarachnoid haemorrhage (aSAH), extracellular haemoglobin (Hb) in the subarachnoid space is bound by haptoglobin, neutralizing Hb toxicity and helping its clearance. Two exons in the *HP* gene (encoding haptoglobin) exhibit copy number variation (CNV), giving rise to HP1 and HP2 alleles, which influence haptoglobin expression level and possibly haptoglobin function. We hypothesized that the *HP* CNV associates with long-term outcome beyond the first year after aSAH.

Methods: The *HP* CNV was typed using quantitative PCR in 1299 aSAH survivors in the Genetics of Subarachnoid Haemorrhage (GOSH) Study, a retrospective multicentre cohort study with a median follow-up of 18 months. To investigate mediation of the *HP* CNV effect by haptoglobin expression level, as opposed to functional differences, we used rs2000999, a single nucleotide polymorphism associated with haptoglobin expression independent of the *HP* CNV. Outcome was assessed using modified Rankin and Glasgow Outcome Scores. SAH volume was defined by the Fisher grade. Haemoglobin-haptoglobin complexes were measured in cerebrospinal fluid (CSF) of 44 aSAH patients, and related to the *HP* CNV.

Results: The HP2 allele associated with a favourable long-term outcome after high-volume, but not low-volume aSAH (multivariable logistic regression). However rs2000999 did not predict outcome. The HP2 allele associated with lower CSF haemoglobin-haptoglobin complex levels. The CSF Hb concentration after high-volume and low-volume aSAH, was respectively higher and lower than the Hb-binding capacity of CSF haptoglobin.

Conclusion: The HP2 allele carries a favourable long-term prognosis after high-volume aSAH. Haptoglobin and the Hb clearance pathway are therapeutic targets after aSAH.

Introduction

Extracellular haemoglobin (Hb) is toxic and is immediately neutralized by the protein haptoglobin (Hp) as a result of a high affinity binding interaction. The Hp-Hb complex is then recognized and endocytosed by the cell surface receptor CD163 [1]. After aneurysmal subarachnoid haemorrhage (aSAH), Hb is released into the cerebrospinal fluid (CSF) from damaged erythrocytes trapped in the subarachnoid space, where it is toxic to neurones and other cells in the central nervous system [2]. The haptoglobin-CD163 Hb clearance mechanism is also present in the central nervous system [3].

The *HP* gene codes for the α and β chain of haptoglobin. Two codominant *HP* alleles exist: HP1 and HP2; the α chain coding region is duplicated in the HP2 allele, so this is a copy number variant (CNV). Three possible *HP* CNV genotypes: HP1-1, HP2-1 and HP2-2, generate the three types of haptoglobin polymers, Hp1-1, Hp2-1 and Hp2-2 [4], illustrated in Figure 1. In HP1-1 individuals, haptoglobin consists of two chains (α1 and β) linked by one disulphide bond. The α1 chain has another free cysteine which leads to dimerization of the haptoglobin molecule, so that the only form present in HP1 homozygotes (HP1-1) is the haptoglobin dimer. In HP2 homozygotes (HP2-2), two free cysteines in the duplicated α2 region endow haptoglobin with the capacity to form cyclic polymers of increasing size. In heterozygotes (HP2-1), linear polymers of increasing size occur, and the dimer is also present.

In several small studies, the *HP* CNV was variably associated with short-term to medium-term outcome after aSAH [5-9], but an individual patient level data analysis did not confirm this [10]. An important consideration is that these studies looked at outcome mostly within the first six months after aSAH, and this may not be early enough to allow early brain injury events other than Hb, to settle. Another unresolved question relates to the mechanism of action. *HP* alleles are associated with differential haptoglobin expression (HP1-1 > HP2-1 > HP2-2 [11]) as well as haemoglobin-haptoglobin complex scavenging rate by CD163 *in vitro* [1 12-14]. It is not clear which of these two consequences of the *HP* CNV mediate its effect on aSAH outcome.

To more definitively address these issues, we studied the effect of the *HP* CNV in the Genetic and Observational Subarachnoid Haemorrhage (GOSH) cohort [15] study of long-term outcome in aSAH survivors, assessed at a median time from ictus of 18 months, up to 8

years. We hypothesized that the *HP* CNV affects long-term outcome after aSAH, and investigated how much of this effect was mediated by haptoglobin expression level using rs2000999, a single nucleotide polymorphism (SNP) associated with haptoglobin expression levels in plasma and tissue (GG > GA > AA), independent of HP CNV [16 17]. The combined use of rs2000999 and the HP CNV is a useful genetic epidemiological tool to dissect the mechanism underlying differences between HP1 and HP2 alleles [18]. We sought mechanistic evidence supporting our findings by performing biochemical analyses in a separate cohort of aSAH patients with available CSF samples.

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 inpatient and outpatient settings following either a new or previous diagnosis respectively; patients who died early after aSAH would not have been 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 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 version [19] of the Rankin Scale [20] 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 mortality [21].

Covariates included age, sex, admission WFNS score [22], admission Fisher grade [23], 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 [24 25], 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.

Genotyping

Detailed genotyping methods for the *HP* CNV and rs2000999 are in the online supplementary methods.

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. Further details are in the online supplementary methods.

We performed haemoglobin-haptoglobin complex quantitation, 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 [26] 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 141.2 [27].

Statistics

Statistical analyses were conducted in R and SPSS v22. For all studies, two-tailed hypotheses were tested with alpha = 0.05. Detailed statistical methods are in the online supplementary methods.

Results

GOSH study cohort

GOSH was a study of long-term outcome in SAH survivors, since patients were assessed after recovery from the acute phase of SAH, with a median time from ictus of 18 months, up to 8 years. A STROBE diagram for the GOSH study participants used in this work is shown in Figure 2. The demographic and clinical characteristics of the GOSH cohort are shown in Table 1 and Supplementary Figure 1.

We considered three essential points to ensure our conclusions are valid. First, because of a potential selection bias toward survivors or those with better functional outcomes in the GOSH study we compared *HP* genotype frequencies in GOSH *versus* a young adult control population (with minimal bias as a result of disease, country of origin, sex and healthcare) from a subset of the ALSPAC (Avon Longitudinal Study of Parents and Children) study, previously genotyped for the *HP* CNV and rs2000999 (n=927). *HP* CNV and rs2000999 genotype frequencies in GOSH were as expected, when compared to ALSPAC (χ^2=2.19, p=0.33 and χ^2=0.39, p=0.82, respectively, Supplementary Table 1). Sex was significantly different between GOSH *versus* ALSPAC (70% *versus* 51% for females respectively, χ^2=81.15, p<0.0001), but there was no sex difference in the *HP* CNV and rs2000999 genotype frequencies in the ALSPAC cohort (χ^2=1.39, p=0.50 and χ^2=2.31, p=0.32, respectively).

Second, although the *HP* CNV and rs2000999 are reported to influence haptoglobin expression levels in other ethnic groups [17 28 29], we confirmed this in a subset of the ALSPAC study in whom the *HP* CNV, rs2000999 and plasma haptoglobin concentration were all available (n=325). In multivariable linear regression, the HP2 allele and rs2000999 A allele were both associated with a similar decrease in plasma haptoglobin of 0.21 and 0.16 g/L respectively (Supplementary Table 2).

Third, since the clinical dataset sample size was smaller (n=907) compared to the whole GOSH cohort (n=1299) (Table 1), we searched for evidence of bias within the GOSH population with clinical data. There was no missingness of any genotype compared to ALSPAC within these 907 patients (*HP* CNV: χ^2=1.262, p=0.53 and rs 2000999: χ^2=0.228, p=0.89, respectively). Moreover the demographic and clinical characteristics of the GOSH participants with available clinical data were similar to those of the whole GOSH cohort (Table 1).

HP genotype and long-term outcome

Next, we investigated the effect of *HP* genotype on favourable functional outcome (defined as modified Rankin scale 0-1) using multivariable logistic regression (Table 2). Favourable outcome was predicted by lower aSAH severity assessed by the clinical World Federation of Neurosurgical Societies score, lower haemorrhage burden as assessed by Fisher category (grades I-II), coiling *versus* clipping, and absence of hydrocephalus, diabetes, hypercholesterolaemia and non-SAH related neurological disability, but not rs2000999. There was a strong interaction between the haemorrhage volume (Fisher category) and the *HP* CNV (Tables 2&3, Figure 3A). *HP* CNV predicted long-term outcome in high Fisher category patients (HP2-2 *versus* HP1-1, Odds ratio of favourable outcome = 2.6, 95% CI 1.4-4.9, p = 0.003), but not low Fisher category patients (Odds ratio = 2.0, 95% CI 0.71-5.6, p = 0.194). On the other hand, the Fisher category predicted long-term outcome in HP1-1, but not in HP2-2 patients (Tables 2&3, Figure 3A). In essence, the poor prognostic effect of a high Fisher category was attenuated by HP2-2, while the Fisher category effect dominated in patients with HP1-1.

There was no evidence of missingness within high or low Fisher category groups that could have biased the results, as shown by several analyses: (1) *HP* CNV genotype frequency was not significantly different between low and high Fisher category groups (χ^2=1.112, p=0.57); (2) *HP* CNV genotype frequency in the high and low Fisher category groups was not significantly different from the ALSPAC control cohort (χ^2=1.685, p=0.43 and χ^2=0.794, p=0.67 respectively); (3) *HP* genotype frequency of patients excluded from the regression due to data availability was not significantly different from that of the included patients (*HP* CNV: χ^2=0.378, p=0.97 and rs 2000999: χ^2=0.288, p=0.87, respectively) or the ALSPAC control cohort (*HP* CNV: χ^2=2.181, p=0.34 and rs 2000999: χ^2=0.562, p=0.76, respectively).

Sensitivity analyses

A similar pattern was confirmed in five sensitivity analyses: (1) using the Glasgow Outcome Scale [30] (Figure 3B); (2) using an alternative dichotomization of the modified Rankin scale, with a favourable outcome defined as 0-2 (Figure 3C); (3) using non-dichotomized Fisher grade (Supplementary Figure 2); (4) using multiple imputation on the whole GOSH cohort (Supplementary Table 4); and (5) analyses across decreasing follow-up intervals (Table 4). The finding that the HP2 allele predicted long-term outcome in high Fisher category patients

was robust to decreasing follow-up time intervals, except at one year. This was not due to smaller sample sizes since the 3-8 epoch had a similar sample size to the ≤ 1 year epoch.

Biochemical studies

Although the *HP* CNV and rs2000999 affect haptoglobin expression level to a similar extent [18], only the *HP* CNV associated with outcome after aSAH, suggesting that functional differences between Hp1-1 and Hp2-2 proteins, perhaps relating to Hb scavenging rather than expression, are likely to be more important. Hence we measured haemoglobin-haptoglobin complexes in serial CSF samples taken from an external ventricular drain after high-grade aSAH (Fisher grade III-IV, n=44, Supplementary Table 3), using ultra-performance size-exclusion liquid chromatography coupled with absorption detection at 415nm. The patients' *HP* CNV status was: HP1-1=9, HP2-1=19, HP2-2=16. All samples contained haemoglobin-haptoglobin complexes, in keeping with saturation of membrane CD163 binding sites in the brain after aSAH, as previously reported [3]. The CSF concentration of haemoglobin-haptoglobin complexes was compared across *HP* CNV types using ANOVA, and was lower in HP2-2 patients than those with HP1-1 (Figure 4A). In an analysis of covariance of CSF haemoglobin-haptoglobin complex concentration across *HP* CNV genotype, controlling for age, sex, clot volume, and CSF/serum albumin quotient, the *HP* CNV genotype was the dominant determinant, explaining 50% of variance in CSF haemoglobin-haptoglobin complex concentration, out of a total of 57% by the whole model (p=0.001, Figure 4B).

The effect of the *HP* CNV on long-term outcome varied with the volume of aSAH. It is known that haptoglobin in the CSF is present at very low concentrations in both healthy controls and after aSAH, such that after high-grade aSAH, haptoglobin is saturated with Hb [3]. We confirmed this observation in our patients; median CSF haptoglobin was 0.29μM (interquartile range: 0.11-0.58μM, expressed as Hb dimer binding capacity) and it was fully saturated with Hb. The low haptoglobin concentration in the CSF has a potential to set up a situation where the system could operate differently depending on Hb concentration. After low-volume aSAH, Hb concentration may be low such that there is sufficient haptoglobin to bind all the Hb, while after high-volume aSAH, the system may be overwhelmed. Ideally one would study haptoglobin saturation with Hb in the CSF from high and low Fisher aSAH patients. However it was challenging to prospectively identify CSF samples from Fisher I-II aSAH patients, since CSF drainage has no place in their clinical management. Nevertheless, we were able to study retrospective data from Fisher I-II aSAH cases referred for

spectrophotometric testing for xanthochromia (median days post-ictus = 2 days, interquartile range 1-3 days, Supplementary Table 5). The median Hb concentration in the CSF of patients with Fisher III-IV aSAH was 2.58µM (interquartile range: 1.07-13.5µM, n=44), i.e. well above the 0.29µM Hb-binding capacity of Hp. In the CSF of patients with Fisher I-II aSAH, the mean Hb CSF concentration was 0.053µM (0.032-0.189µM, n=8), i.e. well below 0.29µM ($p < 0.001$, Figure 4C). These findings provide a potential explanation for the observation that the HP2-2 genotype is only protective after high-volume aSAH.

Discussion

This is the largest study of HP genotype and outcome after SAH, and provides a number of novel insights. The *HP* allele does not associate with outcome after aSAH if this is measured early after aSAH, within the first year [10]. We argue that the *HP* influence on outcome is overshadowed by the effect of early brain injury on outcome in the first year after aSAH, i.e. it takes longer than previously thought for early brain injury effect to settle. In support of this interpretation, we show that the HP2 allele's association with good functional outcome was only detectable two years or more after aSAH (Table 4).

We found that after low-volume aSAH, CSF Hb concentration was within the Hb-binding capacity of CSF haptoglobin, while it was higher in high-volume patients. Hence high-volume patients have unbound Hb available to impact on outcome, so that functional differences between HP genotypes makes a difference after high-volume aSAH.

Collectively, this data suggests that the association of the HP2 allele on long-term outcome after aSAH depends on the haemorrhage burden (Fisher category) and Hb concentration in the CSF. In the presence of high CSF Hb concentration, the HP2 allele is superior to the HP1 allele, being associated with lower haemoglobin-haptoglobin complexes in the CSF and a better functional outcome. At low haemorrhage burden and CSF Hb concentration, the *HP* CNV does not associate with long-term outcome. That the differential clinical effect of the *HP* CNV is mediated via mechanisms other than haptoglobin expression level is supported by the fact that while both the *HP* CNV and rs2000999 associate with haptoglobin expression, only the *HP* CNV is linked to long-term outcome. A recent study has found that lumbar CSF drainage improves outcome in high but not low modified Fisher grade patients [31], which resonates with our findings here.

There is conflicting evidence in the literature regarding the relative efficacy of haptoglobin types in CD163-mediated cellular uptake of haemoglobin-haptoglobin complexes. Although one study suggested that haemoglobin-haptoglobin complex uptake is better with Hp1-1 [12], two subsequent studies have reported that Hp2-2 is better [13 14] which would be in keeping with the results from biochemical binding studies [1 12]. Although the differences between these *in vitro* studies may be due to experimental technicalities, the conflicting results suggest that the difference between the two alleles may not be marked. However it is possible that a subtle difference between HP1 and HP2 allele protein products is amplified in the brain where the low CD163 expression level is a limiting factor in Hb scavenging [3 32]. The low haemoglobin-haptoglobin complex concentration in the CSF of HP2 carriers could be due to lower haptoglobin expression in the CSF, as would be expected for the HP2 allele. However lower CSF haptoglobin levels in HP2 carriers would carry a worse outcome after SAH, not a better one. Also rs2000999 did not associate with outcome. It is therefore more likely that haemoglobin-haptoglobin complex scavenging after high-grade SAH is better in HP2 carriers, *versus* HP1. The higher valency of Hp2-containing complexes likely improves clustering of CD163 receptors [33]. The larger size of the Hb-Hp2-2 complexes (compared to the smaller Hb-Hp1-1 complexes), may also prevent their entry into the brain parenchyma, thereby reducing neurotoxicity. These explanations need further careful study.

The association of the HP2 allele with good long-term outcome in high Fisher grade patients is in contrast to the findings from a mouse model of SAH where HP2-2-transgenic animals had a worse outcome compared to HP1-1 wild-type mice [34]. It is important to bear in mind that there are marked differences in the biochemistry of Hb scavenging between mouse and man. In particular, the haptoglobin receptor CD163 has a higher affinity for haemoglobin-haptoglobin complexes in man, but not in mice [35]. Also, human CD163 is cleaved during inflammation, releasing soluble CD163, but this does not happen with mouse CD163 [36]. For these two reasons, differences in haptoglobin types with respect to CD163 binding are more likely to be important in humans than in mice.

In conclusion, in patients with aSAH who have a high haemorrhage burden, the HP2 allele is associated with favourable long-term functional outcome, possibly via improved haemoglobin-haptoglobin complex clearance. Our findings suggest that preclinical trials of haptoglobin supplementation should consider testing Hp1-1 *versus* Hp2-2. Also, the *HP* CNV genotype and its interaction with Fisher grade should be considered when designing prognostic algorithms and clinical trials in aSAH.

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Author contributions

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Competing Interests:

Nothing to report.

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Table 1. GOSH demographics and clinical characteristics: whole cohort[a] and clinical outcome dataset[b]. Notes: Mean & range[c], number and %[d], median & range[e], % reported is of available data[a] or of total data[b], NA: DNA not available.

	Entire aSAH cohort[a]	**Missingness analysis**[a]	**Outcome analysis**[b]
Number	1729	1299	907
Age (years)[c]	53.2 (12-92)	53 (16-92)	53 (19-92)
Sex[d]			
Male	514 (29.7%)	385 (30%)	261 (29%)
female	1215 (70.3%)	914 (70%)	646 (71%)
WFNS[d]			
1	950 (57.2%)	711 (56.9%)	509 (56%)
2	364 (21.9%)	278 (22.3%)	210 (23.2%)
3	71 (4.3%)	54 (4.3%)	45 (5%)
4	171 (10.3%)	129 (10.3%)	86 (9.5%)
5	104 (6.3%)	77 (6.2%)	57 (6.3%)
Fisher grade[d]			
1	139 (8.9%)	94 (8.0%)	74 (8.1%)
2	466 (29.9%)	363 (30.8%)	280 (30.9%)
3	347 (22.3%)	266 (22.5%)	206 (22.7%)
4	607 (38.9%)	457 (38.7%)	347 (38.3%)
Hydrocephalus[d]			
Present	608 (35.2%)	459 (35%)	324 (36%)
Absent	1121 (64.8%)	840 (65%)	583 (64%)
Aneurysmal management[d]			
Coiled	1367 (79.1%)	991 (78%)	720 (79%)
Clipped	297 (17.2%)	265 (21%)	180 (20%)
Supportive	65 (3.7%)	14 (1%)	7 (1%)
Aneurysm location[d]			
Anterior circulation	1411 (81.61%)	1087 (84%)	774 (85%)
Posterior circulation	211 (12.2%)	177 (14%)	126 (14%)
Not classified	107 (6.19%)	35 (3%)	7 (1%)
Nimodipine[d]			
Administered	1612 (93.2%)	1211 (93%)	870 (96%)
Not administered	117 (6.8%)	88 (7%)	37 (4%)
Time since ictus (months)[e]	15 (0-519)	18 (0-519)	17 (0-96)
Hypertension[d]			
Present	542 (31.4%)	383 (29%)	274 (30%)
Absent	1187 (68.7%)	916 (71%)	633 (70%)
Diabetes mellitus[d]			

Present Absent	69 (4%) 1660 (96%)	53 (4%) 1246 (96%)	34 (4%) 873 (96%)
Smoking (pack-years)[c]	20.9 (0-137)	17 (0-137)	17 (0-137)
Hypercholesterolemia[d]			
Present Absent	350 (20.2%) 1379 (79.8%)	262 (20%) 1026 (80%)	196 (22%) 711 (78%)
Other disability[d]			
Present Absent	116 (7%) 1151 (93%)	92 (7%) 1146 (93%)	64 (7%) 843 (93%)
***HP* CNV genotype**[a]			
HP1-1 HP2-1 HP2-2	NA NA NA	205 (16%) 612 (47%) 481 (37%)	142 (16%) 424 (47%) 341 (37%)
rs2000999 genotype[a]			
AA AG GG	NA NA NA	57 (5%) 379 (29%) 854 (66%)	39 (4%) 270 (30%) 598 (66%)

Table 2. Logistic regression model for primary outcome (favourable mRS 0-1). Logistic regression model fit was excellent (log-likelihood chi-squared test $p<10^{-27}$; Hosmer & Lemeshow test p=0.305). The model explained 32% of the variance in functional outcome. WFNS = World Federation of Neurosurgical Societies; OR = Odds ratio; CI = confidence interval.

	p (overall effect)	**OR**	**95% CI**		**Contrast (*vs* reference)**	**p (contrast)**
Age	0.490	0.995	0.980	1.010		
Sex	0.446	1.156	0.797	1.677	Female (*vs* male)	
WFNS	**<0.001**	4.787	2.404	9.531	WFNS 1 (*vs* 5)	**<0.001**
Hydrocephalus	**<0.001**	2.004	1.386	2.897	Absent (*vs* present)	**<0.001**
Aneurysmal treatment	**0.005**	1.817	1.194	2.763	Coiling *vs* clipping	**0.014**
Nimodipine	0.111	1.914	0.862	4.249	Given *vs* not given	
Followup time	0.121	1.007	0.998	1.015		
Centre	**<0.001**					**<0.001**
Hypertension	0.761	0.951	0.650	1.392	Absent (*vs* present)	
Diabetes	**0.035**	2.529	1.068	5.986	Absent (*vs* present)	
Smoking (pack-years)	0.441	1.003	0.995	1.012		
Hypercholesterolemia	**0.023**	1.636	1.070	2.502	Absent (*vs* present)	
Non-aSAH related disability	**<0.001**	5.536	2.984	10.271	Absent (*vs* present)	
rs2000999	0.359	1.469	0.646	3.341	GG *vs* AA	0.154
Fisher x *HP*	**0.013**					
Fisher	**0.009**	4.105	1.428	11.806	Low *vs* high Fisher in HP1-1	
HP	**0.011**	2.602	1.381	4.904	HP2-2 *vs* HP1-1 at high Fisher	**0.003**

Table 3. The effects of haemorrhage burden (Fisher category) and *HP* CNV on favourable outcome (mRS 0-1) are mutually dependent.

The impact of *HP* CNV on favourable outcome (mRS 0-1) depends on Fisher grade

	n (HP1-1 *versus* HP2-2)	**OR**	**95% C.I.**		**p**
Low Fisher (I-II)	181 (52 *vs* 129)	1.991	0.705	5.628	0.194
High Fisher (III-IV)	302 (90 *vs* 212)	0.384	0.204	0.724	**0.003**

	n (HP2-1 *versus* HP2-2)	**OR**	**95% C.I.**		**p**
Low Fisher (I-II)	302 (173 *vs* 129)	1.433	0.771	2.665	0.255
High Fisher (III-IV)	463 (251 *vs* 212)	0.660	0.418	1.043	0.075

	n (HP2-1 *versus* HP1-1)	**OR**	**95% C.I.**	**p**	
Low Fisher (I-II)	225 (173 *vs* 52)	0.502	0.178	1.419	0.194
High Fisher (III-IV)	341 (251 *vs* 90)	1.718	0.951	3.102	0.073

The impact of Fisher grade on favourable outcome (mRS 0-1) depends on the *HP* CNV

	n (low *versus* high Fisher)	**OR**	**95% C.I.**		**p**
HP1-1	142 (52 *vs* 90)	4.105	1.428	11.806	**0.009**
HP2-2	341 (129 vs 212)	1.262	0.703	2.265	0.435

Table 4. Sensitivity analysis at different follow-up intervals. Findings are largely robust, except at shorter follow-up of one year or less.

A. For mRS 0-1

Sample size	Time since ictus	*HP* CNV[1]	*HP* CNV[1]	rs2000999[2]
		Low Fisher grade	High Fisher grade	
			OR, 95% CI, p value	
907	≤ 8 years	0.5, 0.2-1.4, 0.194	2.6, 1.4-4.9, **0.003**	NS, p = 0.359
863	≤ 6 years	0.4, 0.1-1.2, 0.106	2.8, 1.5-5.4, **0.002**	NS, p = 0.263
776	≤ 4 years	0.5, 0.2-1.5, 0.193	2.8, 1.4-5.4, **0.003**	NS, p = 0.258
575	≤ 2 years	0.5, 0.2-1.7, 0.258	2.0, 1.1-3.4, 0.100	NS, p = 0.807
349	≤ 1 year	0.5, 0.1-2.4, 0.367	1.6, 0.5-5.1, 0.415	NS, p = 0.967
204	≤ 6 months	0.2, 0.02-1.5, 0.114	0.8, 0.1-4.8, 0.777	NS, p = 0.929
87	≤ 3 months	NS, p = 1.0	NS, p = 1.0	NS, p = 1.0
332	3-8 years	0.4, 0.04-4.3, 0.404	4.4, 1.3-14.4, **0.014**	NS, p = 0.215

B. For GOS 5

Sample size	Time since ictus	*HP* CNV[1]	*HP* CNV[1]	rs2000999[2]
		Low Fisher grade	High Fisher grade	
			OR, 95% CI, p value	
907	≤ 8 years	0.3, 0.1-1.0, **0.045**	2.7, 1.4-5.3, **0.003**	NS, p = 0.415
863	≤ 6 years	0.3, 0.1-1.0, **0.047**	2.8, 1.4-5.5, **0.002**	NS, p = 0.472
776	≤ 4 years	0.3, 0.1-1.3, 0.119	2.9, 1.4-5.9, **0.003**	NS, p = 0.532
575	≤ 2 years	0.3, 0.1-1.4, 0.132	2.8, 1.2-6.4, **0.019**	NS, p = 0.627
349	≤ 1 year	0.4, 0.1-2.7, 0.370	2.8, 0.7-10.6, 0.128	NS, p = 0.856
204	≤ 6 months	0.09, 0.01-1.5, 0.91	6.4, 0.5-87, 0.165	NS, p = 0.677
87	≤ 3 months	NS, p = 1.0	NS, p = 1.0	NS, p = 1.0
332	3-8 years	0.0, 0.0-0.0, 0.998	4.0, 1.1-15.1, **0.039**	NS, p = 0.512

[1] HP2-2 *versus* HP1-1, for favourable outcome

[2] rs2000999 G *versus* A, for favourable outcome

Figure legends

Figure 1. Haptoglobin types: Hp1-1, Hp2-1 and Hp2-2. The *HP* gene codes for the α and β chain of Hp. Two codominant *HP* alleles exist: HP1 and HP2; the α chain coding region is duplicated in the HP2 allele, so this is a copy number variant (CNV). Three possible *HP* CNV genotypes: HP1-1, HP2-1 and HP2-2, generate three types of haptoglobin polymers, Hp1-1, Hp2-1 and Hp2-2.

Figure 2. STROBE diagram

Figure 3. HP association with outcome after aSAH depends on clot volume. **A:** The mean predicted probability of favourable outcome (mRS: 0-1) ± standard deviation, by *HP* CNV and Fisher category. The *HP* CNV predicted long-term outcome in high Fisher category patients (HP2-2 *versus* HP1-1, Odds ratio (OR) of favourable outcome = 2.6, 95% CI 1.4-4.9, p = 0.007). In the low Fisher category, there is a trend suggesting that the reverse might be happening (i.e. that HP1-1 confers a favourable outcome *versus* HP2-2), but this was not significant (OR=2.0, 95% CI 0.71-5.6, p = 0.194), despite the lower standard deviations in the low Fisher category. **B:** The mean predicted probability of favourable outcome (GOS: 5) ± standard deviation, by *HP* CNV and Fisher category. At high Fisher grade: p=0.003, OR=2.74 (95% CI: 1.4-5.3) for HP2-2 *versus* HP1-1. At low Fisher grade: p=0.045, OR=0.26 (95% CI: 0.07-0.97) for HP2-2 *versus* HP1-1. **C:** The mean predicted probability of favourable outcome (mRS: 0-2) ± standard deviation, by *HP* CNV and Fisher category. At high Fisher: p=0.002, OR=3.26 (95% CI: 1.5-6.9) for HP2-2 *versus* HP1-1. At low Fisher: p=0.149, OR=0.211 (95% CI: 0.03-1.7) for HP2-2 *versus* HP1-1.

Figure 4. Haptoglobin-haemoglobin complex scavenging in CSF varies with the *HP* CNV in high-grade aSAH. **A.** ANOVA of CSF haemoglobin-haptoglobin complex concentration across *HP* CNV types (n=44, p=0.003, F=6.58, df=43). Post-hoc group comparisons were performed using Bonferroni adjustment. Plot shows means ± standard deviation. **B.** ANCOVA of CSF haemoglobin-haptoglobin complex concentration across *HP* CNV types controlling for age, clot volume, CSF/serum albumin quotient and sex (n=19, p=0.006 and partial eta squared=0.566 for model). We performed group comparisons with Bonferroni adjustment. The plot shows estimated marginal means ± 95% confidence intervals. **C.** CSF Hb concentration in Fisher grade I-II (n=8) and III-IV (n=44). Plot shows medians ± interquartile range. Mann-Whitney U test. Dotted line represents the Hb-binding capacity of haptoglobin in CSF.