Haptoglobin genotype and outcome after spontaneous intracerebral haemorrhage

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43   **Word count**: 3319/3500

44   **Number of references**: 40

45   **Keywords:** Intracerebral haemorrhage, Haptoglobin, intracerebral haemorrhage volume,

46   oedema extension distance, perihaematomal oedema volume, functional outcome, death,

47   ALSPAC

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59 **CONFLICT OF INTEREST**

60 The authors report no conflict of interest concerning the materials or methods used in this study

61 or the findings specified in this paper.

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63 **ACKNOWLEDGEMENTS**

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CONTRIBUTORSHIP STATEMENT

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108　　for intellectual content; obtained funding for the study

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ABSTRACT

Objective: Haptoglobin is a haemoglobin-scavenging protein that binds and neutralises free haemoglobin and modulates inflammation and endothelial progenitor cell function. A *HP* gene copy number variation (CNV) generates HP1 and HP2 allele, the single nucleotide polymorphism rs2000999 influences their levels. HP1 allele is hypothesized to improve outcome after intracerebral haemorrhage (ICH). We investigated the associations of the *HP* CNV genotype and rs2000999 with haematoma volume, perihaematomal oedema (PHO) volume, and functional outcome as well as mortality after ICH.

Methods: We included patients with neuroimaging-proven ICH, available DNA, and six-month follow-up in an observational cohort study (CROMIS-2). We classified patients into three groups according to the *HP* CNV: 1-1, 2-1 or 2-2 and also dichotomized *HP* into HP1-containing genotypes (HP1-1 and HP2-1) and HP2-2 to evaluate the HP1 allele. We measured ICH and PHO volume on CT; PHO was measured by oedema extension distance. Functional outcome was assessed by modified Rankin score (unfavourable outcome defined as mRS 3-6).

Results: We included 731 patients (mean age 73.4, 43.5% female). Distribution of *HP* CNV genotype was: HP1-1 n=132 (18.1%); HP2-1 n=342 (46.8%); and HP2-2 n=257 (35.2%). In the multivariable model mortality comparisons between HP groups, HP2-2 as reference, were as follows: OR HP1-1 0.73, 95%CI 0.34-1.56 (p-value=0.41) and OR HP2-1 0.5, 95%CI 0.28-0.89 (p-value=0.02) (overall p-value=0.06). We found no evidence of association of *HP* CNV or rs200999 with functional outcome, ICH volume or PHO volume.

Conclusion: The HP2-1 genotype might be associated with lower 6-month mortality after ICH; this finding merits further study.

INTRODUCTION

Spontaneous (non-traumatic) intracerebral haemorrhage (ICH) is the most devastating form of stroke with a mortality of about 40% at one month, and 65% at one year[1-3]. Patients who survive frequently remain severely disabled[4]. Moreover, incidence of ICH is increasing in the elderly population[5-7], in part due to increasing use of oral anti-coagulation[5-7].

Spontaneous ICH results from bleeding into the brain parenchyma arising from the rupture of an arterial vessel, most often (>80%) a small arteriole affected by cerebral small vessel diseases (SVD). The commonest sporadic SVD that cause ICH are deep perforator arteriopathy (also termed hypertensive arteriopathy or arteriolosclerosis) and cerebral amyloid angiopathy (CAA). A minority of ICH (less than 20%) is caused by structural or macrovascular bleeding sources such as tumours, arteriovenous malformations, cavernomas or fistulas. Deep perforator arteriopathy is associated with hypertension and is a frequent cause of deep ICH; CAA is caused by amyloid beta deposition in cortical and leptomeningeal blood vessels and is a key cause of lobar ICH.

Haptoglobin is an acute-phase protein which neutralizes free haemoglobin by binding it, and in doing so targets haemoglobin to the CD163 receptor for clearance[8-15]. Haptoglobin prevents the toxic and inflammatory effects of haemoglobin by shielding its iron-containing pocket, and preventing its breakdown into haem and iron, which consequently cause cytotoxicity and brain oedema[8-15]. The *HP* gene has a copy number variant (CNV), which leads to two co-dominant alleles: HP1 and HP2. Three different *HP* CNV genotypes exist: HP1-1, HP2-1 and HP2-2, and their respective protein products differ in molecular size and haemoglobin-binding capacity[15-17]. A previous study demonstrated some evidence that patients with the HP2 allele have a larger haematoma volume, though the underlying mechanisms remain unknown[18]. An increase in haematoma volume may be accompanied by more perihaematomal oedema (PHO)[18 19]. ICH and PHO volume have been demonstrated to influence functional outcome[18]

158 [19]. A previous study reported worse functional outcome for patients with HP2 allele (HP2-1 or

159 2-2) compared to HP1-1 patients as well as some evidence for increased mortality for each

160 HP2 allele[18]. The *HP* CNV might be associated with functional outcome after ICH through

161 differences in haemoglobin clearance and protection from the cytotoxic and inflammatory

162 effects of haemoglobin breakdown products. However most previous studies investigating

163 haptoglobin in ICH are based on investigations in rodents.

164 The single nucleotide polymorphism (SNP) rs2000999 accounts for up to 50% of variation in

165 circulating haptoglobin levels in the blood independently of the *HP* CNV[20]. The combined use

166 of the *HP* CNV and rs2000999 has been suggested as an important genetic tool to discriminate

167 between two potential mechanisms underlying differences between HP1 and HP2 alleles:

168 haptoglobin expression level and functional differences in haptoglobin protein products[21].

169 We performed a comprehensible multivariable study investigating the influence of the *HP*

170 CNV and rs2000999 SNP on functional outcome and mortality after ICH. We also aimed to

171 assess the influence of the *HP* CNV and the rs2000999 SNP on ICH volume and OED.

172

173 **METHODS**

174 **Data collection**

175 We considered patients, of predominantly Caucasian descent, with spontaneous ICH and

176 available blood samples recruited into the Clinical Relevance of Microbleeds in Stroke ICH

177 study[22]. We defined spontaneous ICH as a non-traumatic haemorrhage into the brain

178 parenchyma, presumed due to cerebral SVD after the exclusion of patients with an underlying

179 structural or macrovascular cause.

180 We collected detailed information on demographics, risk factors, medication, clinical

181 presentation, and radiological data. A diagnosis of hypertension, hypercholesterolaemia and

182 diabetes mellitus was present if reported by the patient, stated on medical records or if either

183   drug treatment or any other form of advice (including lifestyle changes) was given. Smoking

184   was defined as current and previous use. All patients had acute brain imaging with CT. Written

185   informed consent was obtained from all participants, or a relative or representative. We

186   excluded patients <18 years, patients without available or adequate CT scan. Patients with a

187   CT scan after 72 hours from symptom onset were excluded from the primary ICH and PHO

188   volume analysis.[18 23 24]. We classified ICH location into lobar, deep (basal ganglia, thalamus),

189   cerebellar and brainstem according to a validated rating scale[25]. Our outcomes were death and

190   functional outcome at 6 months (measured by the modified Rankin Scale [mRS] dichotomized

191   into favorable [mRS 0-2] or unfavorable [mRS 3-6] categories).

192   **Haptoglobin genotyping**

193   To determine the *HP* CNV we optimised a high-throughput qPCR genotyping assay as

194   described previously[26]. The assay amplified a region in the 5` terminal of the *HP* gene's first

195   exon as an internal control (HP5`), and the breakpoint of the HP duplication (HP2). The

196   HP2/HP5` ratio (theoretically either 0, 1, or 2) was used to determine the genotype as HP1-1,

197   HP2-1 or HP2-2 respectively. Samples were run in triplicates; triplicates with a HP2/HP5`ratio

198   coefficient of variation >10% were re-assayed. A second method of *HP* genotyping by PCR[27]

199   was performed on samples with HP2/HP5' ratio values between 0.46-077, in order to confirm

200   the *HP* CNV genotype. Rs2000999 was genotyped using Kompetitive Allele Specific PCR

201   (KASP) assay technology[28] (LGC Genomics Limited, Hertfordshire, UK), call rate was 97.3%.

202   **Measurement of ICH and PHO volume**

203   We measured ICH and PHO volume as previously described via a semi-automated, threshold-

204   based approach[29]. PHO was measured by the oedema extension distance (OED) using a

205   previously described formula[19]; the rationale behind using OED is that PHO extends a

206   consistent mean linear distance from the border of the ICH, independently of its volume.

207

Statistical analysis

We present categorical variables using frequency and percentages, continuous variables using mean ± standard deviation (SD). We transformed ICH and PHO volume with cube root transformation to satisfy statistical normal distribution assumptions. We conducted a *post hoc* sensitivity analysis comparing patients with ICH volume and OED before and after 72 hours. We assessed the distribution of the *HP* CNV and rs2000999 SNP in the CROMIS-2 cohort compared to ALSPAC (Avon Longitudinal Study of Parents and Children) cohort of healthy individuals, which we used as controls. ALSPAC is a general population cohort study[30 31]; *HP* genetic data and rs2000999 SNP data was available from 927 and 748 participants. The ALSPAC study website (http://www.bristol.ac.uk/alspac/researchers/our-data/) contains details of all the data 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. To evaluate the HP1 allele, we also assessed the *HP* CNV as a dichotomized variable (HP1-1 and HP2-1 versus HP2-2) according to our pre-specified analysis plan.

We first performed univariable analyses for each of the four outcomes separately with demographic, clinical and radiological variables of interest. We subsequently fitted multivariable logistic regression models with significant variables from the univariable analysis in addition to pre-specified variables. For the analysis of ICH and OED volume we adjusted the models with the pre-specified variables: time from event to imaging, location of ICH, systolic blood pressure (SBP), *HP* CNV and rs200999 SNP. For functional outcome and mortality analysis, we fitted the multivariable model with the pre-specified variables: age, sex, hypertension, oral anticoagulation (OAC), *HP* CNV and rs200999 SNP. Additionally, we fitted the multivariable models with variables that were statistically significant at the 20% level in the univariable analysis.

233 We investigated whether there were interactions between different variables. However, no

234 interaction reached our pre-specified significant threshold for interactions of p<0.001 (chosen

235 to guard against overfitting) and were therefore not included in the models[32].

236 Statistical analysis was performed using STATA 15 (StataCorp. 2011. *Stata Statistical*

237 *Software: Release 15*. College Station, TX: StataCorp LP).

238

239 **Ethical approval**

240 The CROMIS-2 study was approved by the local Ethics Committee (reference: 10/H0716/64).

241

242 **RESULTS**

243 For the primary analysis of functional outcome at 6 months we included 732 patients. One

244 DNA sample was uncallable for the *HP* CNV and 20 for the rs2000999 SNP. For the secondary

245 analyses of ICH volume and PHO we included 709 patients with an available CT scan (Figure

246 1). OED mas measured at a mean of 10 hours from ICH onset. Patients who were genotyped

247 (n=844) were not different to those without DNA (n=250) with regard to baseline

248 characteristics and risk factor profile (data not shown). The rs2000999 genotype frequency in

249 CROMIS-2 was as expected when compared to ALSPAC (Supplementary Table 1). However,

250 compared to ALSPAC, CROMIS-2 patients less often had the HP2-2 CNV. We found no

251 systematic difference in demographics, comorbidities and ICH characteristics between those

252 with and without available outcome variable (data not shown).

253

254 **Mortality**

255 Of 731 patients with available follow-up and genotype data, 112 died within 6 months (15.3%)

256 and 318 (43.5%) were female.

257 The distribution of the *HP* CNV was 132 HP1-1 (18.1%), 342 HP2-1 (46.8%) and 257 HP2-2

258 (35.2%). Distribution of the SNP allele was: 27 A:A (3.8%), 234 A:G (32.9%) and 451 G:G

259 (63.3%), 20 samples were not callable (2.7%).

260 Patients who died were older, more frequently female, more frequently on OAC, had a lower

261 GCS on admission (GCS <8), a higher ICH and PHO volume, and intraventricular extension

262 (IV). Results of the univariable analysis are shown in supplementary Table 2.

263 The mortality according to *HP* CNV was as follows: HP1-1 18.2%; HP2-1 12.6%; HP2-2

264 17.5%. In the multivariable model (n=608) mortality comparisons between the *HP* groups,

265 with HP2-2 as a reference group, were as follows: OR HP1-1 0.73, 95% CI 0.34-1.56 (p-

266 value=0.41) and OR HP2-1 0.5, 95% CI 0.28-0.89 (p-value=0.02) (overall p-value=0.06, Table

267 1).

268

269 Table 1: Factors associated with 6 month mortality after ICH in an adjusted multivariable
270 logistic regression model
271

	OR	95% CI	P value
Age (years)	1.11	1.07-1.14	<0.001
Female Sex	1.14	0.68-1.92	0.63
Hypertension	1.01	0.57-1.76	0.99
Diabetes mellitus	1.31	0.65-2.65	0.46
Oral anticoagulation	1.25	0.74-2.11	0.4
GCS on admission (binary) - GCS 3-8 - GCS 9-15 (reference)	4.23	1.35-13.28	0.01
ICH location - Cerebellar (reference) - Brainstem - Deep - Lobar	Empty 0.98 0.64	 0.33-2.93 0.2-2	0.38
Cr ICH volume (mL)	2.03	1.48-2.8	<0.001
OED (cm)	2.82	1.01-7.92	0.05
IV extension	1.56	0.89-2.72	0.12
HP CNV - *HP*1-1 - *HP*2-1 - *HP*2-2 (reference)	 0.73 0.5	 0.34-1.56 0.28-0.89	0.06
Rs2000999 - A:A (reference) - A:G - G:G	 0.6 0.58	 0.15-2.36 0.15-2.28	0.74

272
273 cm = centimeter; CNV = copy number variation; Cr = cube root; CT = computed
274 tomography; GCS = Glasgow Coma Scale; *HP* = Haptoglobin; ICH = intracerebral
275 haemorrhage; IV = intraventricular; ml = milliliter; OAC: oral anticoagulation; SBP: systolic
276 blood pressure
277

278   When dichotomizing *HP* into HP1-1/2-1 versus HP2-2 there was evidence for association of

279   decreased mortality with the HP1 allele compared to HP2-2 (OR 0.55, 95%CI 0.31-0.95,

280   p=0.03, supplementary Table 3). As expected, there was also evidence for an increase in

281   mortality with increasing age (OR 1.11, 95%CI 1.07-1.14, p<0.001), decreased GCS on

282   admission <9 (OR 4.37, 95%CI 1.39-13.73, p=0.01), and ICH volume (OR 1.99, 95%CI 1.45-

283   2.74, p<0.001).

284

285   We further investigated the association between mortality and *HP* CNV across tertiles of all

286   the covariates included in the multivariable model as a *post hoc* analysis. Mortality differed

287   between the *HP* groups for older patients (>80 years) with lower (<12.2mL) ICH volume: in

288   this subgroup, mortality was 26% for HP1-1, 14% for HP2-1 and 42% for HP2-2. Patients died

289   at a median of 3.8 months after ICH. There was no difference (early vs. late death) in the time

290   of death after ICH across *HP* CNV or rs2000999 groups, in the overall cohort or the subgroup

291   of >80 years and <12.2mL ICH volume (regression data not shown, supplementary Figure 1).

292   The mortality rate was similar across the *HP* groups for the remaining patients: 15% for HP1-

293   1, 12% for HP2-1 and 12% for HP2-2. The association between mortality and *HP* CNV was

294   confirmed across tertiles of all the other covariates. Finally, we investigated covariates not

295   included in the multivariable model, to see whether they differed across *HP* genotypes, but

296   found no bias to explain the association between mortality and *HP* CNV (data not shown).

297

298   **Functional outcome**

299   Of 731 patients, 444 (60.7%) suffered an unfavourable outcome (mRS 3-6). Dichotomized

300   unfavourable mRS according to *HP* CNV was as follows: HP1-1 64.4%; HP2-1 59.7%; HP2-

301   2 60.3%.

302   Patients with an unfavourable outcome were older, more frequently female, on OAC, more

303   frequently had hypertension, hypercholesterolaemia, presented with a lower GCS (GCS of 3-

304   8), had a higher ICH and PHO volume and IV extension. See supplementary Table 2 for

305   univariable analysis.

306   In the multivariable model (n=623) age (OR 1.04, 1.02-1.06 95%CI; p<0.001), female sex (OR

307   2.31; 1.58-3.37; 95%CI; p<0.001) and the cube root of the ICH volume (OR 1.5; 1.22-1.85

308   95%CI; p<0.001) were significantly associated with functional outcome (Table 2). Neither *HP*

309   CNV nor rs2000999 SNP were associated with functional outcome.

310

311 Table 2: Factors associated with unfavourable outcome after ICH in an adjusted multivariable
312 regression model
313

	OR	95% CI	P value
Age (years)	1.04	1.02-1.06	<0.001
Female Sex	2.31	1.58-3.37	<0.001
Hypertension	1.37	0.92-2.04	0.12
Diabetes mellitus	1.18	0.71-1.97	0.52
Oral anticoagulation	1.16	0.77-1.73	0.49
Antiplatelets	1.08	0.7-1.69	0.72
Hypercholesterolaemia	1.17	0.78-1.75	0.44
GCS on admission (binary) - GCS 3-8 - GCS 9-15 (reference)	3.56	0.76-16.5	0.11
Cr ICH volume (mL)	1.5	1.22-1.85	<0.001
IV extension	1.38	0.9-2.12	0.14
Surgical evacuation	1.84	0.45-7.5	0.39
HP CNV - *HP*1-1 - *HP*2-1 - *HP*2-2 (reference)	 1.17 0.97	 0.67-2.03 0.65-1.45	0.78
Rs2000999 - A:A (reference) - A:G - G:G	 1.19 1.39	 0.43-3.3 0.5-3.84	0.66

314
315
316 CNV = copy number variant; Cr = cube root; CT = computed tomography; GCS = Glasgow
317 Coma Scale; *HP* = Haptoglobin; ICH = intracerebral haemorrhage; IV = intraventricular; ml
318 = millilitre; OAC: oral anticoagulation; SBP: systolic blood pressure
319

320

Intracerebral haemorrhage volume and oedema extension distance

Of the 731 patients included in the functional analysis, 709 had a CT scan available, and of these 68 were >72 hours after symptom onset (Figure 1). Of the remaining 641 individuals, 453 (70.7%) had a scan <24h, 172 (26.8%) between 24-48h and 16 (2.5%) between 48-72h.

See Figure 2 for the association of the *HP* CNV and SNP with OED and ICH volume.

Mean ICH volume was 13.8 mL ($\pm$ 18.82 SD), mean PHO volume 19.54 mL ($\pm$ 20.56 SD) and mean OED 0.51 cm ($\pm$0.23 SD). Variables significantly associated with ICH volume in the univariable analysis are listed in the supplementary Table 3.

In the fitted multivariable model (n=604) ICH location (overall p<0.001) and intraventricular extension (coefficient 0.53; 0.37-0.68; p<0.001) were associated with greater ICH volume (Table 3). Neither *HP* CNV nor the SNP rs2000999 were associated with ICH volume.

Table 3: Factors associated with the cube root ICH volume in an adjusted multivariable regression model

	Coefficient	95% CI	P value
Age (years)	-0.005	-0.01-0.001	0.09
Time Event to CT			0.35
- Day 1 (reference)			
- Day 2	0.04	-0.23-0.31	
- Day 3	-0.29	-0.7-0.11	
ICH location			**<0.001**
- Cerebellar (reference)			
- Brainstem	-0.73	-1.22-0.23	
- Deep	-0.13	-0.44-0.18	
- Lobar	0.79	0.47-1.1	
SBP (mmHg)	0.001	-0.002-0.002	0.88
Platelet level (x10^9/liter)	0.001	-0.0004-0.001	0.31
Hypercholesterolaemia	0.09	-0.05-0.22	0.2
IV extension	0.53	0.37-0.68	<0.001
Neurosurgery	0.36	-0.06-0.78	0.1
HP CNV			0.66
- *HP*1-1	-0.09	-0.25-0.52	
- *HP*2-1	-0.02	-0.17-0.13	
- *HP*2-2 (reference)			
Rs2000999			0.68
- A:A (reference)			
- A:G	0.14	-0.25-0.52	
- G:G	0.16	-0.22-0.54	

CNV = copy number variation; CT = computed tomography; *HP* = Haptoglobin; ICH = intracerebral haemorrhage; IV= intraventricular; mmHg = millimetre mercury; SBP= systolic blood pressure

18

After dichotomizing the *HP* CNV into HP1-1/2-1 versus HP2-2 we did not observe any evidence of an association in univariable or multivariable analyses (p = 0.39 [supplementary Table 4] and p = 0.6 respectively [data not shown]). Similar results were observed when dichotomizing *HP* CNV into HP1-1 versus HP2-1/2-2 [supplementary Table 4].

Oedema Extension Distance

Variables significantly associated with OED in the univariable analysis are listed in supplementary Table 4. For comparison of *HP* CNV and SNP for ICH volume and OED see Figure 2.

In the multivariable linear regression model (n=623), ICH location (with lobar and deep ICH locations featuring a longer OED and with a brainstem location featuring a shorter OED, compared to the reference group of cerebellar location, overall p<0.001) and antihypertensive medication (coefficient -0.09; 95%CI -0.16-(-0.02); p=0.01) were significantly associated with OED (Table 4). Neither the univariable nor multivariable analysis showed evidence of association of *HP* CNV or rs2000999 SNP with OED.

Similar to the ICH volume model, dichotomizing *HP* did not yield any evidence of association in univariable and multivariable models (data not shown).

Table 4: Factors associated with size of oedema extension distance in an adjusted multivariable regression model

	Coefficient	95% CI	*P* value
Female Sex	0.01	-0.02-0.05	0.44
Time Event to CT			0.18
- Day 1 (reference)			
- Day 2	0.07	-0.008-0.14	
- Day 3	0.04	-0.07-0.15	
ICH location			<0.001
- Cerebellar (reference)			
- Brainstem	-0.08	-0.21-0.06	
- Deep	0.16	0.07-0.24	
- Lobar	0.24	0.15-0.33	
SBP (mmHg)	0.0002	-0.0003-0.001	0.49
OAC	0.05	-0.02-0.12	0.17
Antihypertensive medication	-0.09	-0.16-(-0.02)	0.01
Platelet level (x10^9/liter)	0.0002	-0.00005-0.0004	0.11
IV extension	-0.03	-0.07-0.008	0.11
HP CNV			0.5
- *HP*1-1	0.03	-0.02-0.09	
- *HP*2-1	0.01	-0.03-0.05	
- *HP*2-2 (reference)			
Rs2000999			0.93
- A:A (reference)			
- A:G	0.01	-0.09-0.11	
- G:G	0.003	-0.1-0.1	

CNV = copy number variation; CT = computed tomography; *HP* = Haptoglobin; ICH = intracerebral haemorrhage; mmHg = millimetre mercury; OAC: oral anticoagulation; SBP: systolic blood pressure

DISCUSSION

In this large prospective, multicentre cohort study, *HP* was not associated with functional outcome as assessed by the mRS. The *HP* CNV distribution was comparable to that reported in a previous study, apart from a slightly higher proportion of HP1-1 patients and lower proportion of HP2-2[18]. Despite the larger sample size, we could not replicate this previous study's finding of an association of the HP2 allele with functional outcome[18].

However, we found evidence that mortality was lower in HP2-1 patients compared to HP2-2 homozygotes; our *post hoc* analyses suggest that this observation is mostly driven by older patients with lower ICH volumes. No association with mortality was found for the rs2000999 SNP (which is associated with haptoglobin expression level)[21]. This suggests that any link between the *HP* CNV and mortality is mediated by factors other than haptoglobin expression.

While the *HP* CNV's association with mortality could have been confounded by bias in a variable excluded from the model, we did not find any evidence for this. Such a factor could still remain unidentified, but a more likely explanation is that patients who died did not contribute to functional outcome analysis. We found evidence of HP2-2 missingness (of subjects of a particular genotype, in this case HP2-2), when comparing CROMIS-2 with ALSPAC cohorts, which might suggest that the HP2-2 genotype confers a mortality risk.

We confirmed previous results showing evidence towards increased mortality with HP2-2[18], but did not observe a unidirectional dose response of HP alleles in a direction of increasing or decreasing mortality across *HP* genotypes (mortality: HP1-1 18.2%; HP2-1 12.6%; HP2-2 17.5%). The lower mortality in HP2-1 individuals could be a chance finding. A possible but unlikely explanation is heterozygote advantage or heterosis[33]. At a molecular level, the HP1

395 allele might protect against the deleterious effect of the HP2 allele only when the two alleles

396 are present together in HP2-1 individuals. Both HP1 and HP2 alleles scavenge haemoglobin,

397 with HP2 being superior[34][35], and this confers a beneficial effect. However, HP2 has additional

398 off-target effects which are deleterious, mostly pro-inflammatory[36]. In HP2-2 individuals, the

399 better haemoglobin scavenging potential of HP2 versus HP1 is offset by its proinflammatory

400 effects, so that mortality is similar in HP1-1 and HP2-2 individuals. In HP2-1 individuals, the

401 HP1 allele may be negating the deleterious effect of HP2, so that a greater benefit is observed

402 in HP2-1 individuals than is expected by simple co-dominance of the two alleles.

403

404 We did not confirm previous findings of worse functional outcome in patients with HP2 allele,

405 which could be due to the significantly smaller cohort size and statistical power of the previous

406 study, with potential for a chance finding[18].

407

408 PHO develops over a continuous period of time in three main stages. It peaks after two weeks,

409 however its evolution is most rapid in the first 2-3 days[37]. PHO is thought to be mediated by a

410 process of toxicity and inflammation[19][37]. We hypothesized that by modulating neurotoxicity

411 and inflammatory processes haptoglobin might have influenced PHO and functional

412 outcome.[38] However, we did not find any association of *HP* genetic variants (CNV or the

413 rs2000999 SNP) with OED. Similarly, *HP* genetic variants were not associated with ICH

414 volume, which, like haemtoma expansion, is more likely to be driven by other factors including

415 hydrostatic pressure at the bleeding point[18].

416

417 Despite having a large cohort available, we could not replicate the previous study's reported

418 finding of an association of the HP2 allele with larger ICH volumes and IV extension [18]. Since

419 ICH volume and OED was assessed on CT scans performed within 72 hours of symptom onset,

420   we cannot exclude an association of *HP* with ICH volume or OED after this timepoint, although

421   our exploratory analysis of scans beyond 72 hours (n=68) and found no difference in ICH

422   volume and OED across *HP* genotypes (for both CNV and rs2000999 SNP) (data not shown).

423   We found that long-term antihypertensive medication prior to ICH event is independently

424   associated with decreased OED, even after correcting for SBP. It is possible that patients on

425   antihypertensive medication could have reduced sympathetic activity and inflammatory

426   response when ICH occurs[39], a hypothesis that merits further study. As we did not collect

427   follow-up scans, we cannot comment on a potential influence of SBP on haematoma growth.

428

429   Our study has strengths. Our prospective, multi-centre study is the largest on *HP* and ICH to

430   date, and should be generalizable to Caucasian populations. We collected detailed baseline

431   clinical and brain imaging data and undertook multivariable regression analysis adjusting and

432   correcting for important predictors of all four outcomes, and took exceptional care to control

433   for covariates.

434

435   However, our study also has limitations. Since we obtained informed or proxy consent, our

436   study is biased towards ICH survivors with less severe ICH than would be included in an

437   unselected incident ICH population. However, it is likely that any protective effect of *HP* is

438   most relevant in ICH patients who survive the acute period. Additionally, CT scans at multiple

439   timepoints were not available and therefore we could not assess the influence of *HP* CNV and

440   rs200999 SNP on ICH, PHO or OED expansion over time. We also did not have data on the

441   time interval between the ICH and CT scan. However, in a *post hoc* sensitivity analysis ICH

442   volume before and after 72 hours was very similar although OED was larger in patients with

443   first imaging after 72 hours. As PHO increases beyond 72 hours further studies are needed to

444   assess an influence of the *HP* CNV and rs2000999 SNP on oedema expansion. Although we

445　excluded patients without blood samples available for genetic analysis, there were no

446　systematic differences in demographics, comorbidities and ICH characteristics between those

447　with and without genetic data available. Finally, it would have been interesting to study plasma

448　and cerebrospinal fluid haptoglobin levels in relation to *HP* genetic variants, but unfortunately

449　these were not available.

450

451　**CONCLUSION**

452　We investigated the association of *HP* genetic variation (the *HP* CNV and the rs2000999 SNP)

453　in a large cohort of 731 ICH patients. We found evidence in support of a lower mortality with

454　the HP2-1 genotype, but not functional outcome, ICH volume or OED. While *HP* genotype

455　may not matter for functional outcome, upregulating or supplementing haptoglobin may still

456　be of benefit, as demonstrated in animal studies[40], so understanding how different haptoglobin

457　types associate with outcome is important. A future meta-analysis may be appropriate to

458　confirm our observations, and longer follow-up may be needed in case there is an association

459　with longer term outcome.

460

461

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FIGURE LEGENDS

Figure 1. Patient selection flow diagram

Figure 2. A) Differences in OED in Haptoglobin genotype and SNP, B) Differences in ICH volume in Haptoglobin genotype and SNP

Supplementary Figure 1. A) Time to death in days by HP CNV overall cohort, B) Time to death in days by rs2000999 overall cohort, C) Time to death in day by HP CNV subgroup >80 years <12.2mL ICH volume, D) Time to death in day by rs2000999 subgroup >80 years <12.2mL ICH volume