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Haptoglobin, alpha-thalassaemia and glucose-6-phosphate dehydrogenase polymorphisms and risk of abnormal transcranial Doppler among patients with sickle cell anaemia in Tanzania

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Summary

Transcranial Doppler ultrasonography measures cerebral blood flow velocity (CBFv) of basal intracranial vessels and is used clinically to detect stroke risk in children with sickle cell anaemia (SCA). Co-inheritance in SCA of alpha-thalassaemia and glucose-6-phosphate dehydrogenase (G6PD) polymorphisms is reported to associate with high CBFv and/or risk of stroke. The effect of a common functional polymorphism of haptoglobin (HP) is unknown. We investigated the effect of co-inheritance of these polymorphisms on CBFv in 601 stroke-free Tanzanian SCA patients aged <24 years. Homozygosity for alpha-thalassaemia 3/C17 deletion was significantly associated with reduced mean CBFv compared to wild-type (β-coefficient \(-1.70\) cm/s, \(P = 0.002\)) adjusted for age and survey year. Inheritance of 1 or 2 alpha-thalassaemia deletions was associated with decreased risk of abnormally high CBFv, compared to published data from Kenyan healthy control children (Relative risk ratio [RRR] = 0.53 [95% confidence interval (CI):0.35–0.8] & RRR = 0.43 [95% CI:0.23–0.78]), and reduced risk of abnormally low CBFv for 1 deletion only (RRR = 0.38 [95% CI:0.17–0.83]). No effects were observed for G6PD or HP polymorphisms. This is the first report of the effects of co-inheritance of common polymorphisms, including the HP polymorphism, on CBFv in SCA patients resident in Africa and confirms the importance of alpha-thalassaemia in reducing risk of abnormal CBFv.

Keywords: sickle cell disease, Africa, children, cerebral blood flow velocity.

Although sickle cell anaemia (SCA) is a monogenic disorder caused by the homozygous inheritance of sickle haemoglobin (HbS) resulting in haemolytic anaemia, there is a wide variation in the severity and pattern of morbidities (Beutler, 2001). The contributions of other co-inherited genetic variants on risk of severe outcomes such as stroke are under investigation (Sebastiani et al, 2005; Flanagan et al, 2011). Stroke is reported to occur in 11% of patients aged <20 years (Ohene-Frempong et al, 1998; Pandey & Gorelick, 2005). Transcranial Doppler ultrasonography (TCD) is a well-established measure of cerebral blood flow velocity (CBFv) of basal intracranial vessels and abnormal CBFv may be secondary to increased cerebral blood flow or to decrease in diameter of the vessel secondary to vasospasm or stenosis. The technique is used clinically to detect SCA patients with a high risk of stroke (Adams et al, 1992) with velocities of >200 cm/s and >170 cm/s predicting 40% and 7% risk of stroke over the subsequent 3 years without treatment.
(Adams et al, 1992). The risk is substantially reduced by chronic blood transfusion (Adams et al, 1998). Elevated CBFv is also associated with increased risk of other neurological complications, including seizures (Pringle et al, 2005), neurocognitive deficits (Kral et al, 2003; Strouse et al, 2006) and silent infarcts (Pegelow et al, 2001). Low velocities may also be seen in patients with cerebrovascular disease, consistent with proximal vascular stenosis (Kirkham et al, 1986) or occlusion (Lee et al, 2004) and appears to be associated with cerebrovascular accidents (Buchanan et al, 2012).

Haptoglobin (HP) is an acute phase protein that removes free haemoglobin (Hb) from the circulation after haemolysis or tissue damage, and is therefore an important anti-oxidant. HP is encoded by the co-dominant alleles, HP*1 and HP*2, resulting in three distinct phenotypes HP11, HP12 and HP22. Plasma concentrations of HP, and its binding affinities for free Hb, vary by phenotype, with HP22 reported to have increased nitric oxide scavenging by HP2-Hb complexes by macrophages (Asleh et al, 2003). This is suggested to result in increased nitric oxide scavenging by HP2-Hb complexes (Azarov et al, 2008), with reduced downstream anti-inflammatory signalling after macrophage endothytosis of HP2-Hb complexes (Landis et al, 2013), weaker inhibition of prostaglandin synthesis and a stronger angiogenic effect (Cid et al, 1993). There is strong evidence that the HP22 phenotype is associated with increased risk of stroke and cardiovascular events in diabetic patients (Vardi et al, 2012). Furthermore, several reports demonstrate an association between the HP*2 allele with vasospasm after subarachnoid haemorrhage; as determined by high CBFv (Borsody et al, 2006), angiographically (Ohnishi et al, 2013), and in a mouse model (Chaichana et al, 2007). Vasospasm after sub-arachnoid haemorrhage is thought to result from inflammation, oxidative stress, nitric oxide scavenging, leucocyte migration and endothelial-leucocyte interactions (Chaichana et al, 2010), all from extracellular Hb. In HP22 mice with sub-arachnoid haemorrhage, treatment with an anti-oxidant (Froehler et al, 2010) or controlled nitric oxide (Momin et al, 2009) reduces vasospasm, which is supportive evidence that these mechanisms are important. The HP*2 allele is also associated with increased severity and adverse outcomes in various infections including human immunodeficiency virus (HIV and malaria) (Cox et al, 2007; McDermid & Prentice, 2006). Effects of HP phenotype on cerebrovascular outcomes in non-diabetic patients, e.g. those with SCA, have not been reported.

Although the co-inheritance of α-thalassaemia deletions and glucose-6-phosphate dehydrogenase (G6PD) deficiency have been previously investigated as disease modifiers in SCA, specifically affecting risk of elevated CBVf and/or stroke, these have not been investigated in Africa. The co-inheritance of alpha-thalassaemia in SCA modifies red cell indices (Embry et al, 1984; Stevens et al, 1986; Kulozik et al, 1988) and red cell rheology (Serjeant et al, 1983), and, in some reports, increases total Hb (Embry et al, 1982). Alpha-thalassaemia is consistently associated with a decreased risk of increased CBVf and/or stroke in SCA (Adams et al, 1994; Hsu et al, 2003; Bernaudin et al, 2008; Belisario et al, 2010; Flanagan et al, 2011). G6PD deficiency results in decreased capacity to reduce oxidized glutathione via NADPH and thus reduced ability of red cells to counteract oxidant stress (Mason et al, 2007). G6PD deficiency (A-genotype) in SCA is associated with lower Hb but not increased haemolysis (Nouraie et al, 2010). Reports of the effects of co-inheritance of G6PD deficiency with CBVf, vasculopathy on magnetic resonance angiography and/or stroke risk are contradictory (Bernaudin et al, 2008, 2011; Rees et al, 2009; Miller et al, 2011; Thangaraj et al, 2012) and there are no data from patients resident in Africa.

We hypothesized that the co-inheritance of the HP*2 allele in children with SCA would increase the risk of high CBVf and furthermore, that there may be epistatic effects between the three unrelated polymorphisms under investigation such that an effect may be greatest in, or limited to, children with the HP*2 allele who also co-inherited G6PD deficiency and did not have alpha thalassaemia.

Methods

Ethical approval was granted by the Muhimbili University of Health and Allied Sciences, Tanzania (MU/RP/AEC/VOL XI/ 33), and the London School of Hygiene and Tropical Medicine, UK (reference 5158) Review Boards. All participants, parents or guardians gave written informed consent (in Kiswahili) for participation at enrolment into the SCA clinical cohort at Muhimbili National Hospital (MNH).

Study population and Transcranial Doppler examination

Participants aged ≤24 years were enrolled in the Muhimbili Sickle Cohort at Muhimbili National Hospital, Tanzania and had a TCD examination at one or more of three cross-sectional surveys, conducted in 2004/05, 2009 and 2010. Patients who received blood transfusion within the previous two months, symptoms of sickle crisis within the previous 2 weeks or a previous history of stroke were excluded. TCD examinations were performed according to the STOP (Stroke prevention in sickle cell disease) protocol (Nichols et al, 2001) using the Companion II (Nicolet, Warwick, UK). CBVf was determined as the highest time averaged maximum velocity in the distal internal carotid artery and middle cerebral artery on either side. CBVf was classified as low (<43 or <39.5 cm/s) for the left and right middle cerebral artery (LMCA/RMCA), high (>141 or >143.5 cm/s LMCA/RMCA) or normal. CBVf were also classified as conditional (170–199 cm/s) or abnormal (>200 cm/s) based on previous criteria (Adams et al,
1990; Newton et al., 1996). In addition, the difference between the maximum velocities in the ipsilateral to contralateral MCA was calculated; this was previously shown to predict vasospasm in patients after sub-arachnoid haemorrhage (Nakae et al., 2011). The difference between the two sides was then calculated as a percentage of the lower velocity and categorized to indicate the degree of asymmetry between the left and right MCA as: no asymmetry – values less than 40%, degree 1; values 40–75%, degree 2; 75–180%, degree 3; and, >180%, degree 4. These cut-off points are currently used clinically to follow vasospasm after subarachnoid haemorrhage at University Hospital Southampton.

Laboratory procedures

Sickle status was diagnosed and quantification of Hb fractions was performed by high performance liquid chromatography (HPLC) using the β-thalassaemia Short Program on the Variant I analyser (BioRad, Hercules, CA, USA). Full blood counts were performed using an automated cell counter (Pentra 60, Horiba ABX, Kyoto, Japan) within 7 d of the TCD study. Genomic DNA was isolated from peripheral blood leucocytes using Nucleon kits (BACCII). HP functional variants (alleles HP*1 and HP*2) were genotyped by allele-specific polymerase chain reaction (PCR) adapted from published techniques (Koch et al., 2002; Cox et al., 2007). Individuals were genotyped for the 3-7 alpha-thalassaemia deletion using a PCR-based method and agarose gel visualization as per published methods (Williams et al., 2005). The 202- and 376-single nucleotide polymorphisms (SNPs) (rs1050828 [G-202A] & rs1050829 [A-376G]), the combined inheritance of which results in the A- phenotype of glucose 6-phosphate dehydrogenase (G6PD) deficiency, and HbS (rs334) were determined using multiplex Sequenom® Mass-ARRAY® (Sequenom®, Hamburg, Germany).

Statistical analysis and sample size

In the current analysis one observation per subject was selected according to the observation that recorded the highest CBFv, assessed separately in the L- and RMCA. Statistical analyses were performed with Stata IC software (version 12.0, StataCorp, College Station, TX, USA). Categorical variables were constructed for the CBFv values, using the Kenyan and STOP cut-off points as described above. We used two approaches to analyse the CBFv outcome. In the first we used multiple linear regression to explore predictors of CBFv as a continuous variable, whilst excluding those with absent signal. We determined that, using this approach, 90 children per group (HP22 vs. HP11) were required to detect a difference in mean CBFv equivalent to 0.5 × standard deviation (SD) of mean CBFv, based on data from similar aged Kenyan children with SCA (Makani et al., 2009) with 90% power and two-tailed significance at 5%. In the second approach, we used multinomial logistic regression to explore predictors of CBFv as a categorical variable representing potential cardiovascular disease (CVD) (allowing simultaneous investigation of factors affecting risk of low vs. normal and high vs. low CBFv, presenting the results as relative risk ratios (RRRs) with 95% confidence interval (CI). A P-value <0.05 was considered statistically significant.

Results

Characteristics of the study population

A total of 601 homozygous SCA patients were included. The demographic, laboratory and CBFv characteristics of the patients are summarized in Table I. The age of the patients ranged from 0-6 to 22.6 years, with 12% aged <5 years and 2.5% aged >16 years. The majority of subjects (51%) were included from the largest survey in 2004/2005. There was no evidence of a difference between the surveys for sex, genotype or Hb concentrations. However, mean age and HbF% were significantly lower in the 2004/05 survey. All the participants had genotype results for at least one of the three polymorphisms under investigation, with 385 having complete data for the three genes being investigated.

Mean CBFv was 131 cm/s (SD = 42). The proportion of non-detectable CBFv in the LMCA compared to the RMCA was significantly different (P < 0.006) at 5% for the LMCA and 8% for the RMCA. However, there was no significant difference in mean CBFv between the left and right MCA (120 cm/s [SD 42, N = 571] vs. 119 cm/s [SD 42, N = 552]).

The genotype prevalence data are summarized in Table II. The prevalence of the inherited combinations of the three genes in the 385 participants with a complete dataset is available as supplementary material in Table SI.

Factors associated with TCD outcomes

In the first approach we used linear regression to investigate possible associations with CBFv for age, sex, Hb and HbF%, as well as the three genes under investigation, including all measurable CBFv observations. The results are summarized in Table III. Strong inverse associations with CBFv were apparent for age and Hb. Initial analyses of the genotypes were adjusted for age and year of survey. Hb concentration was not immediately adjusted for, as all of the genotypes under investigation may potentially act, at least in part, through effects on Hb. Inheritance of two copies of the 3-7 α-thalassaemia deletion, compared to no copies, was significantly associated with decreased mean CBFv. If Hb was adjusted for, no effect of α-thalassaemia was observed, suggesting the effect is mediated via Hb. There were no apparent effects of G6PD status or HP genotype, even when limited to those with gene combinations hypothesized as the ‘worst’ and ‘best combinations ([HP22/A-A-or A-Z, and/or, 3α/αα] vs. [HP11/BB/-α/-α]).
In the second approach we explored associations using multinomial logistic regression, in which CBFv was categorized as low, normal or high (Table IV). The only genotype with evidence of an association was the 3.7 α-thalassaemia deletion, the inheritance of which was associated with a significantly decreased risk of having an abnormally high or low CBFv.

We also investigated genotype associations with abnormal CBFv according to the STOP classifications (normal, mild, moderate or severe) of asymmetry in CBFv, classified as normal, mild, moderate or severe, respectively. In this analysis, asymmetry was defined as the difference in the maximum velocities in the left and right MCA maximum velocity, left or right, n (%).

| Demographic variables | Summary statistic | Observations (N) |
|-----------------------|-------------------|-----------------|
| Age [years], mean (SD) | 9.76 (3.86)       | 601             |
| Males, n (%)           | 325 (54.08)       | 601             |
| Survey, n (%)          |                   |                 |
| 2004/05                | 305 (50.75)       | 601             |
| 2009                   | 224 (37.27)       | 601             |
| 2010                   | 72 (11.98)        | 601             |
| Laboratory variables* |                   |                 |
| Haemoglobin [g/l], mean (SD) | 74.0 (11.2) | 583             |
| Fetal haemoglobin [%], mean (SD) | 5.23 (3.84) | 533             |
| CBFv                   |                   |                 |
| MCA maximum velocity, left or right, n (%) |                   |                 |
| Normal                 | 534 (88.85)       |                 |
| Low [≤43 cm/s or <39.5 cm/s] | 44 (7.32) |                 |
| High [>141 cm/s or >143.5 cm/s] | 170 (28.29) |                 |
| STOP criteria          |                   |                 |
| Normal                 | 534 (88.85)       |                 |
| Conditional >170 <200 cm/s | 25 (4.16) |                 |
| Abnormal ≥200 cm/s     | 42 (6.99)         |                 |
| Asymmetry (Vasospasm)‡ n (%) |                   |                 |
| No asymmetry           | 317 (54.75)       | 579             |
| Moderate asymmetry     | 104 (17.96)       |                 |
| Severe asymmetry       | 93 (16.06)        |                 |

Table II. Genotype prevalence and scores assigned to each genotype.

| Gene | Homozygote WT | Heterozygote | Homo/hemizygote mutant |
|------|---------------|--------------|------------------------|
| 6GPD A-Phenotype (202 & 376) N = 583 | BB [75-30%] | A-B [11-32%] | A-Z or A-A-[13-38%] |
| 3.7 Alpha-thalassaemia deletion N = 568 | α/α [34-66%] | α/-α [40-67%] | α/-α [15-67%] |
| HP N = 422 | HP11 [23-46%] | HP12 [56-64%] | HP22 [19-91%] |

Table III. Associations between demographic, laboratory variables and genotypic CBFv at the STOP classification (normal, mild, moderate, severe) of asymmetry in CBFv, classified as normal, mild, moderate or severe; SD, standard deviation; CBFv, cerebral blood flow velocity; SCA, sickle cell anaemia; STOP, stroke prevention in sickle cell disease; MCA: middle cerebral artery.

In the second approach we explored associations using multinomial logistic regression, in which CBFv was categorized as low, normal or high (Table IV). The only genotype with evidence of an association was the 3.7 α-thalassaemia deletion, the inheritance of which was associated with a significantly decreased risk of having an abnormally high or low CBFv.

We also investigated genotype associations with abnormal CBFv according to the STOP classifications (normal, mild, moderate, severe) of asymmetry in CBFv, classified as normal, mild, moderate or severe, respectively. In this analysis, asymmetry was defined as the difference in the maximum velocities in the left and right MCA maximum velocity, left or right, n (%).

Table II. Genotype prevalence and scores assigned to each genotype.

| Gene                | Homozygote WT | Heterozygote | Homo/hemizygote mutant |
|---------------------|---------------|--------------|------------------------|
| 6GPD A-Phenotype    |               |              |                        |
| 202 & 376 N = 583   |               |              |                        |
| 3.7 A1α thalassaemia deletion N = 568 |               |              |                        |
| HP N = 422          |               |              |                        |

Discussion

This is the largest study to date to assess the effects of the disease modifying and commonly co-inherited polymorphisms of α-thalassaemia and G6PD on prospectively measured CBFv in SCA, and the first in children and adolescents resident in Africa. In addition, this is the first study to assess the effect of co-inheritance of HP polymorphisms on a clinical end-point in SCA. We confirm previous reports of a reduction in mean CBFv and protection from abnormal CBFv measurements in heterozygote and homozygotes for <170 cm/s, conditional 10–199 cm/s and abnormal ≥200 cm/s (Adams et al, 1998) but no significant associations were observed.

Finally we also investigated possible associations with the degree of asymmetry in CBFv, classified as normal, mild, moderate or severe. No consistent effects were observed for any of the variables tested (Table V).

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Table IV. Relative risk ratios of genetic variants on CBFv categories defined as low or high compared to normal using data from Kenyan healthy non-SCA child population adjusted for age and year of survey.

| Genotypes                        | Relative risk ratio (95% confidence interval)* | N  |
|----------------------------------|------------------------------------------------|----|
|                                  | (CBFv Low vs. normal)                           |    |
| Haptoglobin, HP11                |                                                 |    |
| HP12                             | 0.90 (0.33–2.47)                                | 422|
| HP22                             | 1.01 (0.30–3.38)                                |    |
| α-Thalassaemia, normal           |                                                 |    |
| 1 Deletion                        | 0.38 (0.17–0.83)†                                | 568|
| 2 Deletions                      | 0.95 (0.41–2.20)                                |    |
| G6PD Normal phenotype            |                                                 |    |
| Mild                             | 0.49 (0.14–1.65)                                | 583|
| Affected                         | 0.41 (1.12–1.37)                                |    |

*Low CBFv corresponds to values <43 in LMCA or <39.5 in RMCA, including values of 0; High CBFv corresponds to values >141.1 in LMCA or >143.6 in RMCA), compared to normal (CBFv: 43–141 cm/s in LMCA or 39.6–143.5 cm/s in RMCA)(Newton et al, 1996).
†P-value <0.05.

RRA: Relative risk ratio; CBFv, cerebral blood flow velocity; SCA, sickle cell anaemia; RMCA, right middle cerebral artery; LMCA, left middle cerebral artery.

Table V. Relative risk ratios of genetic variants on category of vascular asymmetry as an indicator of vasospasm adjusted for age and year of survey.

| Genotypes                        | Relative risk ratio (95% confidence interval)* | N  |
|----------------------------------|------------------------------------------------|----|
|                                  | Mild vs. normal                                 |    |
|                                  | Moderate vs. normal                             |    |
|                                  | Severe vs. normal                               |    |
| Haptoglobin, HP11                |                                                 |    |
| HP12                             | 0.08 (0.02–0.14)§                               | 525|
| HP22                             | 0.01 (0.01–0.50)                                |    |
| α-Thalassaemia, normal           |                                                 |    |
| 1 Deletion                        | 0.03 (0.09–0.04)                                |    |
| 2 Deletions                      | 0.01 (0.03–0.04)                                |    |
| G6PD Normal phenotype            |                                                 |    |
| Mild                             | 0.10 (0.10–0.13)                                |    |
| Affected                         | 0.27 (0.00–0.30)†                               |    |

*Degree of asymmetry is difference in the maximum velocities in LMCA and RMCA calculated as a percentage of the lower velocity. Mild = 40–75%, moderate 75–80%, severe >80%.
†P-value <0.05; Results for multivariable regression including the marked variables.
‡P-value <0.05; Adjusted for survey year.
effect of alpha-thalassaemia was apparent for degree of asymmetry between the ipsilateral and contralateral MCA. The effect of the alpha-thalassaemia deletion may be mediated at least in part via increased Hb. Co-inheritance of the 3-7 deletion is consistently and quantitatively associated with decreased mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and haemolytic markers, including in this population, (Cox et al, 2013) and with decreased WBC (Bernaudin et al, 2008). HbS concentration is the primary determinant of HbS polymerization (Eaton & Hofrichter, 1987). Thus the protective effect of co-inheritance of the 3-7 deletion has been commonly assumed to be due to decreased sickling rates (Ballas, 2001) and consequent haemolysis. However, in the analysis by Bernaudin et al (2008) there were independent effects on CBFv of the 3-7 deletion after adjustment for MCV (MCH & MCHC not analysed), white blood cell count and the haemolytic marker lactate dehydrogenase (LDH) (itself independently associated in the multivariate model). The authors suggested increased red cell deformability as a potential mechanism. In a recent, large, carefully controlled study, co-inheritance of the 3-7 deletion in SCA was quantitatively and negatively associated with the proportion of dense dehydrated red cells (%DRBCs) measured at steady state. Increased %DRBC was, in turn, positively associated with risk of priapism, leg ulcers and renal dysfunction, of which only renal dysfunction was associated with haemolysis (Bartolucci et al, 2012). There is evidence to suggest that the increased rigidity of DBRCs may promote vasoconstriction via reduced shear stress-induced ATP release as a vasodilatory signalling molecule (Wan et al, 2008).

It is unlikely that the lack of an effect of the HP*2 allele was due to a type II error as a result of a lack of power, as evidenced by our adequate sample size of 83 HP22 and 103 HP11 compared to our sample size estimate of 90 per group to detect an effect equivalent to half a standard deviation (SD) in CBFv. Thus although there was a probable increased variance in our data due to different observers between survey years, a similar effect size was observed for alpha-thalassaemia in this study (α/α- (N = 94) vs. αα/αα (N = 258), β-coefficient −16.14 cm/s = 0.4 SD of CBFv). However, the 95% CIs of the observed effect of HP22 did just include the effect size in the sample size calculation (−15.3 cm/s vs. 0.5 × SD = 20.9). Thus, it remains a possibility that small effects of the HP*2 allele exist, reducing risk of either both or the lower extremes of CBFv, not captured in the multinomial logistic model, which was not included in pre-study sample size calculations. Thus despite the strong evidence for a mechanism whereby the HP22 variant could be expected to increase vascular activation and vasoconstriction in the inflammatory and haemolytic condition of SCA, our data does not support this suggestion. An explanation for this could be that HP is overwhelmed in SCA, such that phenotypic variations in its functions cease to be relevant. This scenario is supported by our observation that during well, steady-state clinic visits, HP levels were non-detectable in 96% of our patients (lower limit of detection = 0.04 g/l; N = 838) compared to 17% of non-SCA controls and not affected by genotype (S. E. Cox, J. Makani & A. M. Prentice, unpublished data). A possible alternative explanation is that in HP22 individuals the haem-oxygenase-1 compensatory pathway may be more successfully up-regulated in response to haem exposure, demonstrated to have powerful anti-inflammatory and anti-stasis effects (Jison et al, 2004; Belcher et al, 2006).

Conflicting reports of an effect of G6PD on CBFv (Bernaudin et al, 2008, 2011; Rees et al, 2009; Miller et al, 2011; Thangarajh et al, 2012) may result from variations between populations in either the phenotypic expression compared to assessed genotype or from population or methodological differences between in-vivo vs. in-vitro enzyme activity, when this has been measured directly (Johnson et al, 2009). There is little evidence for an association of either low enzyme activity (Miller et al, 2011) or genotype (Flanagan et al, 2011) with stroke. Decreased enzyme activity was associated with TCD abnormality in children resident in France (Bernaudin et al, 2008) and this effect appeared to be independent of haemolysis. However, this association was not observed in England (Rees et al, 2009). Genotypes rs1050828 or rs1050829 were associated with magnetic resonance angiography evidence of vasculopathy in a cohort from the US, England and France but children with TCD abnormality were excluded (Thangarajh et al, 2012). It is possible that G6PD polymorphisms play a role in the initiation or development of vasculopathy in certain environments but we have no evidence for this in our population resident in Africa and the lack of an association with stroke suggests that other risk factors may be more important.

In conclusion, despite adequate power to determine a clinically relevant effect size of the HP*2 allele on CBFv, we could determine no evidence of such an effect. In a population of SCA patients resident in Africa, we confirmed the effect of alpha-thalassaemia, but could not find an effect of G6PD.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. Proportions of subjects for each combination of the three genotypes under investigation.

References

Adams, R.J., Nichols, F.T. 3rd, Azulay, R., McKie, V.G., McKie, K., Carl, E., Stephens, S., Thompson, W.O., Milner, P. & Figueroa, R. (1990) Cerebral vesel stenosis in sickle cell disease: criteria for detection by transcranial Doppler. The American Journal of Pediatric Hematology/ Oncology, 12, 277–282.

Adams, R.J., Nichols, F.T., Figueroa, R., McKie, V. & Lott, T. (1992) Transcranial Doppler correlation with cerebral angiography in sickle cell disease. Stroke, 23, 1073–1077.

Adams, R.J., Kutlar, A., McKie, V., Carl, E., Nichols, F.T., Liu, J.C., McKie, K. & Clary, A. (1984) Alpha thalassemia and stroke risk in sickle cell anemia. American Journal of Hematology, 45, 279–282.

Adams, R.J., McKie, V.C., Hsu, L., Files, B., Vichinsky, E., Pegelow, C., Abboud, M., Gallacher, D., Kutlar, A., Nichols, F.T., Bonds, D.R. & Brambilla, D. (1998) Prevention of a first stroke by transfusions in children with sickle cell anemia and abnormal results on transcranial Doppler ultrasonography. New England Journal of Medicine, 339, 5–11.

Adams, R.J., Brambilla, D.J., Granger, S., Gallacher, D., Vichinsky, E., Abboud, M.R., Pegelow, C.H., Woods, G., Rohde, E.M., Nichols, F.T., Jones, A., Luden, J.P., Bowman, L., Igagnn, S., Morales, K.H. & Roach, E.S. (2004) Stroke and conversion to high risk in children screened with transcranial Doppler ultrasound during the STOP study. Blood, 103, 3689–3694.

Ash, M., Marshall, S., Skilkrut, M., Binah, O., Guetta, J., Lejkowicz, F., Enav, B., Shekeda, N., Kanter, Y., Lache, O., Cohen, O., Levy, N.S. & Levy, A.P. (2003) Genetically determined heterogeneity in hemoglobin scavenging and susceptibility to diabetic cardiovascular disease. Circulation Research, 92, 1193–1200.

Azarov, L., He, X., Jeffers, A., Basu, S., Ucer, B., Hantgan, R.R., Levy, A. & Kim-Shapiro, D.B. (2008) Rate of nitric oxide scavenging by hemoglobin bound to haptoglobin. Nitric Oxide, 18, 296–302.

Ballas, S.K. (2001) Effect of alpha-globin genotype on the pathophysiology of sickle cell disease. Pediatric Pathology & Molecular Medicine, 20, 107–121.

Bartolucci, P., Brugnara, C., Teixeira-Pinto, A., Pisard, S., Moradkhani, K., Jouault, H. & Galactatos, F. (2012) Erythrocyte density in sickle cell syndromes is associated with specific clinical manifestations and hemolysis. Blood, 120, 3136–3141.

Belcher, J.D., Mahaseth, H., Welch, T.E., Otterbein, L.E., Hebbel, R.P. & Vercellotti, G.M. (2006) Heme oxygenase-1 is a modulator of inflammation and vaso-occlusion in transgenic sickle mice. Journal of Clinical Investigation, 116, 808–816.

Belisario, A.R., Rodrigues, C.V., Martins, M.L., Silva, C.M. & Viana, M.B. (2010) Coinheritance of alpha-thalassemia decreases the risk of cerebrovascular disease in a cohort of children with sickle cell anemia. Hemoglobin, 34, 516–529.

Bernaudin, F., Verlhac, S., Chevet, S., Torres, M., Coic, I., Arnaud, C., Kamdem, A., Hau, I., Grazia Neonato, M. & Delacourt, C. (2004) Impact of early transcranial Doppler screening and intensive therapy on cerebral vasculopathy outcome in a newborn sickle cell anemia cohort. Blood, 113, 1130–1140; quiz 1436.

Beutler, E. (2001) Discrepancies between genotype and phenotype in hematology: an important frontier. Blood, 98, 2597–2602.

Bersody, M., Burke, A., Coplin, W., Miller-Lotan, R. & Levy, A. (2006) Haptoglobin and the development of cerebral artery vasospasm after subarachnoid hemorrhage. Neurology, 66, 634–640.

Buchanan, I.D., James-Herry, E. & Osunkwo, I. (2004) Concurrent sickle cell anemia and alpha-thalassemia: Effect on pathological properties of sickle erythrocytes. Journal of Clinical Investigation, 73, 116–123.

Flanagan, J.M., Frohlich, D.M., Howard, T.A., Schultz, W.H., Driscoll, C., Nagasubramanian, R., Mortier, N.A., Kimble, A.C., Aygun, B., Adams, R.J., Helms, R.W. & Mare, E.P. (2011) Genetic predictors for stroke in children with sickle cell anemia. Blood, 117, 6681–6684.

Froehlich, M.T., Kooshkabadi, A., Miller-Lotan, R., Blum, S., Sher, S., Levy, A. & Tamargo, R.J. (2010) Vasospasm after subarachnoid hemorrhage in haptoglobin 2-2 mice can be prevented with a glutathione peroxidase mimetic. Journal of Clinical Neuroscience, 17, 1169–1172.

Hsu, L.L., Miller, S.T., Wright, E., Kutlar, A., McKie, V., Wang, W., Pergaw, C.H., Driscoll, C., Hurlet, A., Woods, G., Elsas, L., Embury, S. & Adams, R.J. (2003) Alpha Thalassemia is associated with decreased risk of abnormal transcranial Doppler ultrasonography in children with sickle cell anemia. Journal of Pediatric Hematology/Oncology, 26, 622–628.

Jison, M.L., Munson, P.J., Barb, J.J., Sufredini, A.F., Talwar, S., Logun, C., Raghaavachari, N., Beigel, J.H., Shelhamer, H., Danner, R.L. & Gladwin, M.T. (2004) Blood mononuclear cell gene expression profiles characterize the oxidant, hemolytic, and inflammatory stress of sickle cell disease. Blood, 104, 270–280.

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Johnson, M.K., Clark, T.D., Njama-Meya, D., Rosenthal, P.J. & Parikh, S. (2009) Impact of the method of G6PD deficiency assessment on genetic association studies of malaria susceptibility. PLoS One, 4, e7246.

Kirkham, F.J., Neville, B.G. & Levin, S.D. (1986) Bedside diagnosis of stenosis of middle cerebral artery. Lancet, I, 797–798.

Koch, W., Latz, W., Eichinger, M., Roguin, A., Levy, A.P., Schomig, A. & Kastrati, A. (2002) Genotyping of the common haplotype Hp 1/2 polymorphism based on PCR. Clinical Chemistry, 48, 1377–1382.

Kral, M.C., Brown, R.T., Nietert, P.J., Abboud, M.R., Jackson, S.M. & Hyn, G.W. (2003) Transcranial Doppler ultrasonography and neurocognitive functioning in children with sickle cell disease. Pediatrics, 112, 324–331.

Kulooz, A.E., Kar, C.B., Serjeant, G.R., Serjeant, B.E. & Weatherall, D.J. (1988) The molecular basis of alpha thalassemia in India. Its interaction with the sickle cell gene. Blood, 71, 467–472.

Landis, R.C., Philippidis, P., Domín, J., Boyle, J.J. & Haskard, D.O. (2013) Haptoglobin genotype-dependent anti-inflammatory signaling in CD163(+ ) macrophages. International Journal of Inflammation, 2013, 980327.

Langlois, M.R. & Delanghe, J.R. (1996) Biological and clinical significance of haplotype poly- morphism in humans. Clinical Chemistry, 42, 1589–1600.

Lee, Y.S., Jung, K.H. & Roh, J.K. (2004) Diagnosis of moyamoya disease with transcranial Doppler sonography: correlation study with magnetic resonance angiography. Journal of Neuroimaging, 14, 319–323.

Makani, J., Kirkham, F.J., Komba, A., Ajala-Agbo, T., Otieno, G., Fegan, G., Williams, T.N., Marsh, K. & Newton, C.R. (2009) Risk factors for high cerebral blood flow velocity and death in Kenyan children with Sickle Cell Anemia: role of hemoglobin oxygen saturation and febrile illness. British Journal of Haematology, 145, 529–532.

Mason, P.J., Bautista, J.M. & Gilsanz, F. (2007) G6PD deficiency: the genotype-phenotype association. Blood Reviews, 21, 267–283.

McDermid, J.M. & Prentice, A.M. (2006) Iron and infection: effects of host iron status and the iron-regulatory genes haplotype and NRAMP1 (SLC11A1) on host-pathogen interactions in tuberculosis and HIV. Clinical Science (London), 110, 503–524.

Melamed-Frank, M., Lache, O., Enav, B.I., Szafra- nek, T., Levy, N.S., Rickleis, R.M. & Levy, A.P. (2001) Structure-function analysis of the antioxidant properties of haplotype. Blood, 98, 3693–3698.

Miller, S.T., Milton, J. & Steinberg, M.H. (2011) G6PD deficiency and stroke in the CASSCD. American Journal of Hematology, 86, 331.

Momin, E.N., Schwaab, K.E., Chachkina, K.L., Miller-Lotan, R., Levy, A.P. & Tamargo, R.J. (2009) Controlled delivery of nitric oxide inhibits leukocyte migration and prevents vasospasm in haptoglobin 2-2 mice after subarachnoid hemorrhage. Neurosurgery, 65, 937–945; discussion 945.

Nakae, R., Yokota, H., Yoshiida, D. & Teramoto, A. (2011) Transcranial Doppler ultrasonography for diagnosis of cerebral vasospasm after aneurysmal subarachnoid hemorrhage: mean blood flow velocity ratio of the ipsilateral and contralateral middle cerebral arteries. Neurosurgery, 69, 876–883; discussion 883.

Newton, C.R., Marsh, K., Peshu, N. & Kirkham, F.J. (1996) Perturbations of cerebral hemodynamics in Kenyans with cerebral malaria. Pediatric Neurology, 15, 41–49.

Nichols, F.T., Jones, A.M. & Adams, R.J. (2001) Stroke prevention in sickle cell disease (STOP) study guidelines for transcranial Doppler testing. Journal of Neuroimaging, 11, 354–362.

Nouraei, M.R., Reading, N.S., Campbell, A., Minniti, C.P., Rana, S.R., Luchtman-Jones, L., Kato, G., Gladwin, M.T., Castro, O.L., Prchal, J.T. & Gor- druk, V.R. (2010) Association of G6PD with lower haemoglobin concentration but not increased haemolysis in patients with sickle cell anaemia. British Journal of Haematology, 150, 218–225.

Ohene-Frempong, K., Weiner, S.J., Sleeper, L.A., Miller, S.T., Embury, S., Moohr, J.W., Wethers, D.L., Pegelow, C.H. & Gill, F.M. (1998) Cerebrovascular accidents in sickle cell disease: rates and risk factors. Blood, 91, 288–294.

Ohnishi, H., Iihara, K., Kaku, Y., Yamauchi, K., Fukuda, K., Nishimura, K., Nakai, M., Satow, T., Nakajima, N. & Ikegawa, M. (2013) Hapto- globin phenotype predicts cerebral vasospasm and clinical deterioration after aneurysmal subarachnoid hemorrhage. Journal of Stroke and Cerebrovascular Diseases, 22, 520–526.

Pandey, D.K. & Gorelick, P.B. (2005) Epidemiology of stroke in African Americans and Hispanic Americans. Medical Clinics of North America, 89, 739–752, vii.

Pegelow, C.H., Wang, W., Granger, S., Hsu, L.L., Vichinsky, E., Moser, F.G., Bello, J., Zimmerman, R.A., Adams, R.J., Brambilla, D. & Trial, S. (2001) Silent infarcts in children with sickle cell anemia (SCA). Blood, 98, 16437. Rees, D.C., Lambert, C., Cooper, E., Bartram, J., Goss, D., Deane, C. & Thein, S.L. (2009) Glu- cose 6 phosphate dehydrogenase deficiency is not associated with cerebrovascular disease in children with sickle cell anemia. Blood, 114, 742–743; author reply 743–744.

Sebastiani, P., Ramoni, M.F., Nolan, V., Baldwin, C.T. & Steinberg, M.H. (2005) Genetic dissec- tion and prognostic modeling of overt stroke in sickle cell anemia. Nature Genetics, 37, 435–440.

Sepulveda, B.E., Mason, K.P.,新闻网, M.W., Stuart, J., Higgs, D.R., Weatherall, D.J., Hayes, R.J. & Serjeant, G.R. (1983) Effect of alpha thalassaemia on the rheology of homozygous sickle cell disease. British Journal of Haematology, 55, 479–486.

Stevens, M.C., Mauder, G.H., Beckford, M., Grand- ison, Y., Mason, K., Taylor, B., Serjeant, B.E., Higgs, D.R., Teli, H. & Weatherall, D.J. (1986) Alpha thalassemia and the hematology of homozygous sickle cell disease in childhood. Blood, 67, 411–414.

Strome, J.J., Cox, C.S., Melheim, E.R., Lu, H., Kraut, M.A., Razumovskiy, A., Yohay, K., van Zijl, P.C. & Casella, J.P. (2006) Inverse correlation between cerebral blood flow measured by continuous arterial spin-labeling (CASL) MRI and neurocognitive function in children with sickle cell anemia (SCA). Blood, 108, 379–381.

Thangaraj, M., Yang, G., Fuchs, D., Ponsioso, M.R., McKinstry, R.C., Jau, A., Noetzel, M.J., Casella, J.F., Barron-Casella, E., Hoover, W.C., Boulet, S.L., Bean, C.J., Pyle, M.E., Payne, A.B., Driggers, J., Trau, H.A., Vendt, B.A., Rodheger, M. & DeBaun, M.R. (2012) Magnetic resonance angiography-defined intracranial vasculopathy is associated with silent cerebral infarcts and gluc- cose-6-phosphate dehydrogenase mutation in children with sickle cell anemia. British Journal of Haematology, 159, 352–359.

Vardi, M., Blum, S. & Levy, A.P. (2012) Haploglo- bin genotype and cardiovascular outcomes in diabetes mellitus - natural history of the disease and the effect of vitamin E treatment. Meta- analysis of the medical literature. European Journal of Internal Medicine, 23, 628–632.

Wan, J., Ristenpart, W.D. & Stone, H.A. (2008) Dynamics of shear-induced ATP release from red blood cells. Proceedings of the National Academ- y of Sciences of the United States of America, 105, 16432–16437.

Williams, T.N., Mwanigi, T.W., Wambua, S., Petto, T.E., Weatherall, D.J., Gupta, S., Recker, M., Freeman, B.S., Uyoga, S., Macharia, A., Mwacharo, J.K., Snow, B.W. & Marsh, K. (2005) Negative epistasis between the malaria- protective effects of alpha-thalassemia and the sickle cell trait. Nature Genetics, 37, 1253–1257.