Genome wide association studies of silent cerebral infarction in sickle cell disease (HbSS and HbSC)

by John N. Brewin, Helen Rooks, Kate Gardner, Harry Senior, Mrinmayi Morje, Hamel Patel, David Calvet, Pablo Bartolucci, Swee-Lay Thein, Stephan Menzel, and David C. Rees

Haematologica 2020 [Epub ahead of print]

Citation: John N. Brewin, Helen Rooks, Kate Gardner, Harry Senior, Mrinmayi Morje, Hamel Patel, David Calvet, Pablo Bartolucci, Swee-Lay Thein, Stephan Menzel, and David C. Rees.
Genome wide association studies of silent cerebral infarction in sickle cell disease (HbSS and HbSC).
Haematologica. 2020; 105:xxx
doi:10.3324/haematol.2020.265827

Publisher's Disclaimer.
E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication. E-publishing of this PDF file has been approved by the authors. After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in print on a regular issue of the journal. All legal disclaimers that apply to the journal also pertain to this production process.
Letter to the editor

**Genome wide association study of silent cerebral infarction in sickle cell disease (HbSS and HbSC)**

Authors: John N. Brewin\(^1\), Helen Rooks\(^1\), Kate Gardner\(^1,3\), Harry Senior\(^1\), Mrinmayi Morje\(^1\), Hamel Patel\(^1\), David Calvet\(^4\), Pablo Bartolucci\(^4\), Swee-Lay Thein\(^5\), Stephan Menzel\(^4\)*, David C. Rees\(^1,2\)*

*these authors contributed equally to this paper

Affiliations
1. Kings College London, UK
2. Kings College Hospital, London, UK
3. Guys and St Thomas Hospital, London, UK
4. Henri Mondor Hospital, Paris, France
5. NIH, Bethesda, U.S.A.

Corresponding Author:
Dr John Brewin
Red cell biology group
Rayne Institute,
Kings College London
SE5 9NU

John.brewin@kcl.ac.uk

Word Count: 1473 (Max 1500)
Figures & tables: 3 (Max 3)
References: 15 (Max 15)
Supplementary figures and tables: 2 (Max 3)
Silent cerebral infarcts (SCI) are common in patients with sickle cell disease (SCD). Up to 35% of children with HbSS will have an SCI by the age of 15 years, and this prevalence has been shown to increase linearly with age(1). The exact nature of SCIs is unknown, although they are probably small regions of ischemic damage detectable on MRI. By definition, they do not cause overt neurological deficit. They have, however, been demonstrated to predict a lower IQ and also carry a higher risk of large vessel territory ischemic stroke(2). Established risk factors for SCI in patients with HbSS include a lower baseline Hb, male sex and relative hypertension(3), but there is no consensus on the effect of HbF levels(3-6). Less is known about SCIs in those with HbSC genotype, however, the prevalence in children has been reported at between 5.8-13.5%(7, 8). We performed a retrospective analysis of 333 patients with HbSS and 76 patients with HbSC. We found SCIs occurred far younger in HbSS, with a hazard ratio of 3.01 against HbSC for SCIs, however, the prevalence of SCIs in our HbSC cohort was unexpectedly high at 55%. We also showed that alpha thalassemia and female gender offered protection against SCIs in patients with HbSS, but not HbSC. Additionally, we found no influence of G6PD deficiency on SCIs, and no influence of measured HbF levels, or genetic loci known to influence HbF levels, on SCI outcomes.

Patient data came from the South East London sickle gene bank (London, UK). Written informed consent was obtained through 3 approved study protocols (LREC 01-083, 07/H0606/165, and 12/LO/1610) and research conducted in accordance with the Helsinki Declaration (1975, as revised 2008). Genotyping data were established for 15 million variants using the Illumina Infinium MEGA chip and imputation using 1000genome phase 3 data on the Michigan imputation server as described previously.(9) Alpha thalassemia was determined using single tube Multiplex PCR according to previously published methods.(9) Clinical notes and neuroimaging results from 2000 onwards were reviewed for all patients. Evidence of SCIs were determined by MRI using the accepted neuroradiological criteria(3) and confirmed to have no correlating overt clinical event in the clinical notes. The age at which the first radiological evidence that an SCI had occurred was recorded. Controls were determined by MRI confirming the absence of any white matter hyperintensities. The age was defined by the most recent neuroimaging scan confirming this absence. Kaplan-Meier plots and Cox-proportional hazard (coxPH) ratios were calculated in R 3.6.1. Linear mixed modelling was performed using GCTA, with a genetic relatedness matrix to account for
population structure. Age, gender, sickle genotype and alpha thalassemia were used as covariates. The threshold for genome wide statistical significance was set at 5x10^-8.

The cohort consisted of 333 patients with HbSS and 76 with HbSC genotypes. The average age was 35.8yrs (11.4-78.1yrs) in the HbSS cohort and 52.3yrs (17.6-84.2yrs) in the HbSC cohort. Heterozygous alpha thalassemia (αα/-α3.7) was detected in 130 (32%) of the total cohort, and homozygous alpha thalassemia (-α3.7/-α3.7) in 21 (5%). The prevalence of SCI in those with HbSC was equivalent to that seen in the SCA cohort (53.4% vs 55%), although, as demonstrated in figure 1a, these occurred at a much later age (average age 50.6yrs vs 25.7yrs). CoxPH ratios showed an HR of 3.01 for SCIs in patients with HbSS than those with HbSC.

Our cohort had a slight excess of females (245) to males (164). The Kaplan-Meier plots (Figure 1b) and coxPH ratios demonstrate that males carried a higher risk for SCIs (HR=1.54, 95%CI 1.18-2.03, p=0.0016). Considering the two sickle genotypes individually, shown in Figure 2a and b, we found this to only be a risk factor in patients with HbSS (HR=1.86, 95%CI 1.24-2.8, p=0.002), but not in those with HbSC (HR=0.77, 95%CI 0.38-1.6, p=0.465). G6PD assay results were available for 321 of our cohort, including 36 cases. Adding this as a covariate did not improve the model, and G6PD deficiency was not a statistically significant variable (HR=1.11, 95%CI 0.67-1.8, p=0.69). We further tested this in just the male subgroup and reached the same conclusion.

Alpha thalassemia (AT) is a known protective factor with respect to large vessel cerebrovasculopathy in SCD, however, its effect on SCIs was not known. We report an overall protective influence (HR 0.77 0.6-0.99, p=0.038) on SCI occurrence. Again, we found that this influence was only seen in those with HbSS (HR=0.74, 95%CI 0.56-0.96, p=0.026), but not those with HbSC (HR=0.91 95% CI 0.50-1.7, p=0.774).

We also considered clinical measurements of HbF%. Methods of collection are detailed in a separate study(10). 359 patients had validated HbF measurements. The average HbF% in the HbSS cohort was 7.2% (n=292), and 1.9% (n=67) in those with HbSC. We found no
association between HbF% and SCI outcomes, after adjusting for age, sex, and sickle genotype (OR=0.80, 95%CI=0.51-1.09, p=0.126).

We used our variant dataset to perform genome wide analysis on this patient cohort, using age at defined outcome, sex, sickle genotype and alpha thalassemia as covariates. We also included a genetic relatedness matrix to control for population substructure and cryptic relatedness. The discovery cohort included 403 patients with full phenotype and covariate datasets. The $\lambda_{GC}$ (0.986) and QQ plot (supplementary figure 1a) showed no evidence of genomic inflation. The Manhattan plot (supplementary figure 1b) did not show any variants approaching the threshold of statistical significance. The top 5 variant loci from the analysis are shown in supplementary table 1. We used the summary statistics generated by this analysis to interrogate the association of 5 variants previously reported to affect SCI outcomes(11-13). Additionally, we looked at the variants known to strongly influence HbF levels in sickle cell populations(14). No variants demonstrated an association with SCI at a nominal significance of $p<0.05$ (table 1). Additionally, we evaluated the HbF genetic prediction score, $g$(HbF), which combines 4 markers to form a composite score of the genetic influence on HbF levels(10). This again did not show an association with SCI outcomes. We also confirmed all these negative findings in the HbSS cohort alone.

In this study, we have reviewed prevalence rates of SCIs in patients with sickle cell disease and considered genetic risk factors that may influence their occurrence. We found the SCI prevalence in the HbSS cohort similar to that reported previously(1), but additionally, report that the HbSC patients have a notably high prevalence, albeit at an older age. These data add to the rates reported in childhood studies(7, 8) and suggests that as with HbSS, there is a linear increase in prevalence with age. Moreover, although our HbSC cohort is small in size, our analysis suggests the risk factors are different to those in HbSS. We were unable to explore whether older age risk factors such as diabetes mellitus or hypertension were contributing to SCI risk in this older cohort.

We report, for the first time, the protective effect of alpha thalassemia against the development of SCIs in patients with HbSS. A previous study failed to find an association, although this was a smaller study with less well defined neuroradiological criteria(15). This
protective effect may be related to the higher steady state hemoglobin levels associated with AT, which has previously been shown to confer a two-fold protective effect (<76g/L vs >86g/L)\(^3\). However, there may also be an additional rheological benefit in the form of improved RBC deformability and reduced hemolysis reducing microinfarcts. Unfortunately, we did not have sufficient data on baseline hemoglobin levels in this cohort to assess the interaction of alpha thalassemia and hemoglobin on SCIs.

Our study had some important negative findings. Some studies have found low HbF levels to be a risk factor\(^4-6\) for SCIs, whereas others have not\(^3, 15\). In our cohort, we did not see any association of HbF\% with SCI outcomes. We also did not see an association with the genetic modulators of HbF, nor the composite \(g(HbF)\) prediction score\(^10\), suggesting genetic variation of HbF levels in our population of predominantly west African and Caribbean patients does not determine the risk of SCI. However, we did not consider the possible confounding influence of concurrent large vessel vasculopathy on SCI, which has been suggested to represent an alternative pathogenic mechanism of SCIs\(^4\). Additionally, although we confirmed the increased risk with male sex previously reported\(^3\), we did not find any association of the X-linked condition G6PD deficiency. We also did not find a correlation with candidate variants previously identified. Finally, our own genome wide analysis also did not generate novel candidates, although it is possible that genetic associations might be found by larger studies.

In summary, our key findings are that co-inheritance of alpha thalassemia and female sex, but not elevated HbF\%, provide protection against development of SCIs in patients with HbSS. SCIs are common and under recognised in patients with HbSC, and further studies are needed to better understand the prevalence rates and risk factors in this condition.
References


Table 1. Results from linear mixed modelling on the influence of candidate variants reported to associate with SCIs and variants known to significantly influence clinical HbF levels on SCI outcomes in all patients with SCD, and in those with HbSS genotype.

<table>
<thead>
<tr>
<th>Gene</th>
<th>RS id</th>
<th>All patients</th>
<th>HbSS only</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCAM1</td>
<td>rs1041163</td>
<td>OR=1.08, p=0.675</td>
<td>OR=1.19, p=0.413</td>
</tr>
<tr>
<td>ADAMTS10</td>
<td>rs4275799</td>
<td>OR=0.91, p=0.563</td>
<td>OR=0.89, p=0.511</td>
</tr>
<tr>
<td>NOM1</td>
<td>rs887614</td>
<td>OR=0.99, p=0.944</td>
<td>OR=1.02, p=0.919</td>
</tr>
<tr>
<td>FMD4A</td>
<td>rs3750882</td>
<td>OR=1.12, p=0.456</td>
<td>OR=1.07, p=0.705</td>
</tr>
<tr>
<td>CACNB2</td>
<td>rs2357790</td>
<td>OR=0.79, p=0.081</td>
<td>OR=0.76, p=0.073</td>
</tr>
<tr>
<td>BCL11a</td>
<td>rs654816</td>
<td>OR=1.1, p=0.529</td>
<td>OR=1.04, p=0.791</td>
</tr>
<tr>
<td>BCL11a</td>
<td>rs1427407</td>
<td>OR=0.8, p=0.159</td>
<td>OR=0.85, p=0.374</td>
</tr>
<tr>
<td>BCL11a</td>
<td>rs11886868</td>
<td>OR=0.83, p=0.215</td>
<td>OR=0.89, p=0.508</td>
</tr>
<tr>
<td>HBS1L-MYB</td>
<td>rs9376090</td>
<td>OR=1.88, p=0.347</td>
<td>OR=2.31, p=0.275</td>
</tr>
<tr>
<td>HBS1L-MYB</td>
<td>rs66650371</td>
<td>OR=0.87, p=0.674</td>
<td>OR=0.89, p=0.755</td>
</tr>
<tr>
<td>HMI1P</td>
<td>rs9399137</td>
<td>OR=0.87, p=0.674</td>
<td>OR=0.89, p=0.755</td>
</tr>
<tr>
<td>HMI1P</td>
<td>rs9389269</td>
<td>OR=1.14, p=0.714</td>
<td>OR=1.18, p=0.664</td>
</tr>
<tr>
<td>HMI1P</td>
<td>rs9402686</td>
<td>OR=1.2, p=0.592</td>
<td>OR=1.25, p=0.549</td>
</tr>
<tr>
<td>HMI1P</td>
<td>rs9494142</td>
<td>OR=0.91, p=0.684</td>
<td>OR=0.91, p=0.722</td>
</tr>
<tr>
<td>HMI1P</td>
<td>rs9494145</td>
<td>OR=1.01, p=0.98</td>
<td>OR=1.22, p=0.593</td>
</tr>
<tr>
<td>g(HbF)</td>
<td></td>
<td>OR=1.36, p=0.466</td>
<td>OR=1.08, p=0.487</td>
</tr>
</tbody>
</table>

Figure 1 Survival analysis of factors affecting SCI events in patients with sickle cell disease. A: Kaplan-Meier plot comparing outcomes in HbSS and HbSC genotypes. B: Kaplan-Meier plot comparing outcomes in males and females. C: Kaplan-Meier plots comparing outcomes with no alpha thalassaemia, heterozygous and homozygous of deletional alpha. D: Forest plot of Cox-proportional hazard ratios for the three factors affecting SCI outcomes in patients with sickle cell disease.

Figure 2. Survival analysis of factors affecting SCI events in patients with HbSS and HbSC disease separately. A) Kaplan-meier plot comparing outcomes in males and females in HbSS. B) Kaplan-meier plot comparing outcomes in males and females in HbSC. C) Kaplan-meier plot outcomes with no alpha thalassaemia, heterozygous and homozygous of deletional alpha in patients with HbSS. D) Kaplan-meier plot outcomes with no alpha thalassaemia, heterozygous and homozygous of deletional alpha in patients with HbSS. E) Forest plot of the cox-proportional hazard ratios of the factors affecting SCI outcomes in patients with HbSS. F) Forest plot of the cox-proportional hazard ratios of the factors affecting SCI outcomes in patients with HbSC.
A  Genotype  
- Hb SS  
- Hb SC  

B  Sex  
- Male  
- Female  

C  Deletional alpha thalassemia  
- None  
- Heterozygous  
- Homozygous  

D  Hazard ratios for silent cerebral infarcts  

<table>
<thead>
<tr>
<th>Effect</th>
<th>Reference</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>Hb SC (N=76)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCA (N=333)</td>
<td>Male (N=164)</td>
<td>3.01</td>
<td>(2.1 - 4.36)</td>
<td>&lt;0.001 ***</td>
</tr>
<tr>
<td>Sex</td>
<td>Female (N=245)</td>
<td>1.55</td>
<td>(1.2 - 2.03)</td>
<td>0.002 **</td>
</tr>
<tr>
<td>Alpha_thal</td>
<td>Male (N=409)</td>
<td>0.77</td>
<td>(0.6 - 0.99)</td>
<td>0.038 *</td>
</tr>
</tbody>
</table>

# Events: 218; Global p-value (Log-Rank): 5.4518e-12
AIC: 2061.78; Concordance Index: 0.69
Silent Cerebral Infarcts in patients with Hb SS

A

Sex
Male - Red
Female - Teal

Patients without SCI (%)
0.00 0.25 0.50 0.75 1.00
Age (Years)
0 10 20 30 40 50 60

p = 0.00011

Silent Cerebral Infarcts in patients with Hb SC

B

Sex
Male - Red
Female - Teal

Patients without SCI (%)
0.00 0.25 0.50 0.75 1.00
Age (Years)
0 10 20 30 40 50 60

p = 0.67

Deletional alpha thalassemia

C

None - Red
Heterozygous - Green
Homozygous - Blue

Patients without SCI (%)
0.00 0.25 0.50 0.75 1.00
Age (Years)
0 10 20 30 40 50 60

p = 0.046

D

Deletional alpha thalassemia

None - Red
Heterozygous - Green
Homozygous - Blue

Patients without SCI (%)
0.00 0.25 0.50 0.75 1.00
Age (Years)
0 10 20 30 40 50 60

p = 0.97

Hazard ratios for silent cerebral infarcts in patients with HbSS

E

Sex
Female - (N=190) reference
Male - (N=143) 1.76 (1.30 - 2.37)

p = <0.001

Alpha_thal
(N=333) 0.74 (0.56 - 0.99)

0.026 *

Hazard ratios for silent cerebral infarcts in patients with HbSC

F

Sex
Female - (N=55) reference
Male - (N=21) 0.77 (0.38 - 1.6)

p = 0.465

Alpha_thal
(N=76) 0.91 (0.50 - 1.7)

0.774

# Events: 177; Global p-value (Log-Rank): 6.8208e-05
AIC: 1658.58; Concordance Index: 0.63

# Events: 41; Global p-value (Log-Rank): 0.74363
AIC: 246.36; Concordance Index: 0.85
Supplementary Figure 1: Results of genome wide association testing of SCI outcomes in patients with sickle cell disease. Age, gender, sickle genotype and alpha thalassemia were used as covariates, and a genetic relatedness included to control for population structure. The QQ plot (A) demonstrates no genomic inflation which would be suggestive of population stratification bias. The Manhattan plot (B) demonstrates the p values of the variants analysed. The blue line represents 1x10^-5, whilst the red line represents 5x10^-8 which was used as the threshold of statistical significance.

Supplementary table 1 The top five variants identified in the GWAS described in supplementary figure 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>rsID</th>
<th>Change</th>
<th>MAF</th>
<th>OR</th>
<th>p</th>
<th>Variant location</th>
<th>Gene Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHSY3</td>
<td>rs1557759</td>
<td>G&gt;A</td>
<td>0.222</td>
<td>0.44</td>
<td>1.88E-06</td>
<td>Upstream variant</td>
<td>Glycosyltransferase</td>
</tr>
<tr>
<td>PHACTR2</td>
<td>rs6930487</td>
<td>G&gt;A</td>
<td>0.388</td>
<td>1.94</td>
<td>1.96E-06</td>
<td>Intronic</td>
<td>Platelet response to cytosolic Ca^{2+}</td>
</tr>
<tr>
<td>None</td>
<td>rs201658643</td>
<td>G&gt;GTA</td>
<td>0.318</td>
<td>2.08</td>
<td>2.34E-06</td>
<td>Intergenic</td>
<td>NA</td>
</tr>
<tr>
<td>TOX4</td>
<td>rs10142478</td>
<td>A&gt;C</td>
<td>0.067</td>
<td>0.25</td>
<td>2.76E-06</td>
<td>Intronic</td>
<td>Chromatin Binding</td>
</tr>
<tr>
<td>PRKACB</td>
<td>rs2250806</td>
<td>A&gt;G</td>
<td>0.415</td>
<td>0.51</td>
<td>2.88E-06</td>
<td>Intronic</td>
<td>Mediates cAMP-dependent signalling</td>
</tr>
</tbody>
</table>