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

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 SCI is unknown, although they are probably small regions of ischemic damage detectable on magnetic resonance imaging (MRI). By definition, they do not cause overt neurological deficit. They have, however, been demonstrated to predict a lower intelligence quotient (IQ) and also carry a higher risk of large vessel territory ischemic stroke.[2] Established risk factors for SCI in patients with hemoglobin (Hb) SS 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 SCI 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 SCI occurred far younger in HbSS, with a hazard ratio of 3.01 against HbSC for SCI, however, the prevalence of SCI in our HbSC cohort was unexpectedly high at 55%. We also showed that α thalassemia (AT) and female sex offered protection against SCI in patients with HbSS, but not HbSC. Additionally, we found no influence of glucose-6-phosphate dehydrogenase (G6PD) deficiency on SCI, 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 three 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 1,000genome phase 3 data on the Michigan imputation server as described previously.[9] AT was determined using single tube multiplex polymerase chain reaction (PCR) according to previously published methods.[9] Clinical notes and neuroimaging results from the year 2000 onwards were reviewed for all patients. Evidence of SCI 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-proportionate hazard (coxPH) ratios were calculated in R 3.6.1. Linear mixed modeling was performed using genome-wide complex trait analysis (GCTA), with a genetic relatedness matrix to account for population structure. Age, sex, sickle genotype and AT 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 35.8 years (yrs) (range, 11.4-78.1 yrs) in the HbSS cohort and 52.3 yrs (range, 17.6-84.2 yrs) in the HbSC cohort. Heterozygous AT ($\alpha\alpha/-\alpha^{3.7}$) was detected in 130 (32%) of the total cohort, and homozygous AT ($-\alpha^{3.7}/-\alpha^{3.7}$) 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 50.6 yrs *vs.* 25.7 yrs). CoxPH ratios showed a hazard ratio (HR) of 3.01 for SCI in patients with HbSS than those with HbSC.

Table 1. Results from linear mixed modelling on the influence of candidate variants reported to associate with silent cerebral infarcts (SCI) and variants known to significantly influence clinical HbF levels on SCI outcomes in all patients with sickle cell disease, and in those with HbSS genotype.

Gene	RS id	All patients	HbSS only
VCAM1	*rs1041163*	OR=1.08, *P*=0.675	OR=1.19, *P*=0.413
ADAMTS10	*rs4275799*	OR=0.91, *P*=0.563	OR=0.89, *P*=0.511
NOM1	*rs887614*	OR=0.99, *P*=0.944	OR=1.02, *P*=0.919
FRMD4A	*rs3750882*	OR=1.12, *P*=0.456	OR=1.07, *P*=0.705
CACNB2	*rs2357790*	OR=0.79, *P*=0.081	OR=0.76, *P*=0.073
BCL11a	*rs6545816*	OR=1.1, *P*=0.529	OR=1.04, *P*=0.791
BCL11a	*rs1427407*	OR=0.8, *P*=0.159	OR=0.85, *P*=0.374
BCL11a	*rs11886868*	OR=0.83, *P*=0.215	OR=0.89, *P*=0.508
HBS1L-MYB	*rs9376090*	OR=1.88, *P*=0.347	OR=2.31, *P*=0.275
HBS1L-MYB	*rs66650371*	OR=0.87, *P*=0.674	OR=0.89, *P*=0.755
HMIP	*rs9399137*	OR=0.87, *P*=0.674	OR=0.89, *P*=0.755
HMIP	*rs9389269*	OR=1.14, *P*=0.714	OR=1.18, *P*=0.664
HMIP	*rs9402686*	OR=1.2, *P*=0.592	OR=1.25, *P*=0.549
HMIP2b	*rs9494142*	OR=0.91, *P*=0.684	OR=0.91, *P*=0.722
HMIP2b	*rs9494145*	OR=1.01, *P*=0.98	OR=1.22, *P*=0.593
g(HbF)		OR=1.36, *P*=0.466	OR=1.08, *P*=0.487

RS id: reference single nucleotide polymorphisms identity; HbSS: hemoglobin SS; OR: overall response.

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 SCI (HR=1.54, 95% Confidence Interval [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 with G6PD deficiency. 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.

AT is a known protective factor with respect to large vessel cerebrovasculopathy in SCD, however, its effect on SCI was not known. We report an overall protective influence (HR=0.77, 95%CI, 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] Three hundred fifty-nine 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 (overall response [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 AT as covariates. We also included a genetic relatedness matrix to control for pop-

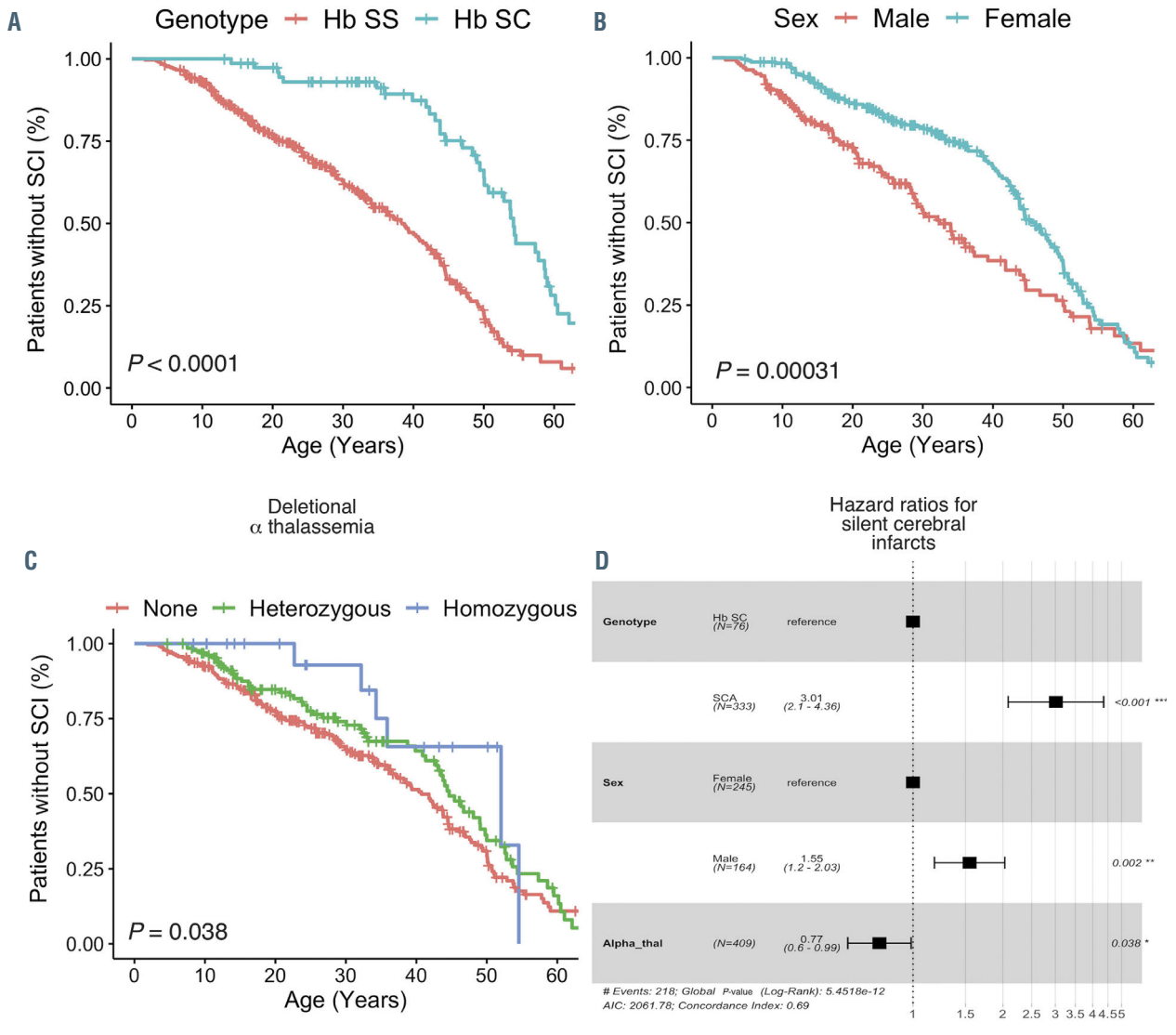

Figure 1. Survival analysis of factors affecting silent cerebral infarcts events in patients with sickle cell disease. (A) Kaplan-Meier plot comparing outcomes in hemoglobin (Hb) SS and HbSC genotypes. (B) Kaplan-Meier plot comparing outcomes in males and females. (C) Kaplan-Meier plots comparing outcomes with no α thalassaemia (AT), heterozygous and homozygous deletional AT. (D) Forest plot of Cox-proportionate hazard ratios for the three factors affecting silent cerebral infarcts outcomes in patients with sickle cell disease.

ulation substructure and cryptic relatedness. The discovery cohort included 403 patients with full phenotype and covariate datasets. The λ_{GC} (0.986) and QQ plot (*Online Supplementary Figure S1A*) showed no evidence of genomic inflation. The Manhattan plot (*Online Supplementary Figure S1B*) did not show any variants approaching the threshold of statistical significance. The top five variant loci from the analysis are shown in the *Online Supplementary Table S1*. We used the summary statistics generated by this analysis to interrogate the association of five 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 four 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 SCI 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 AT against the development of SCI 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 Hb levels associated with AT, which has previously been shown to confer a 2-fold protective

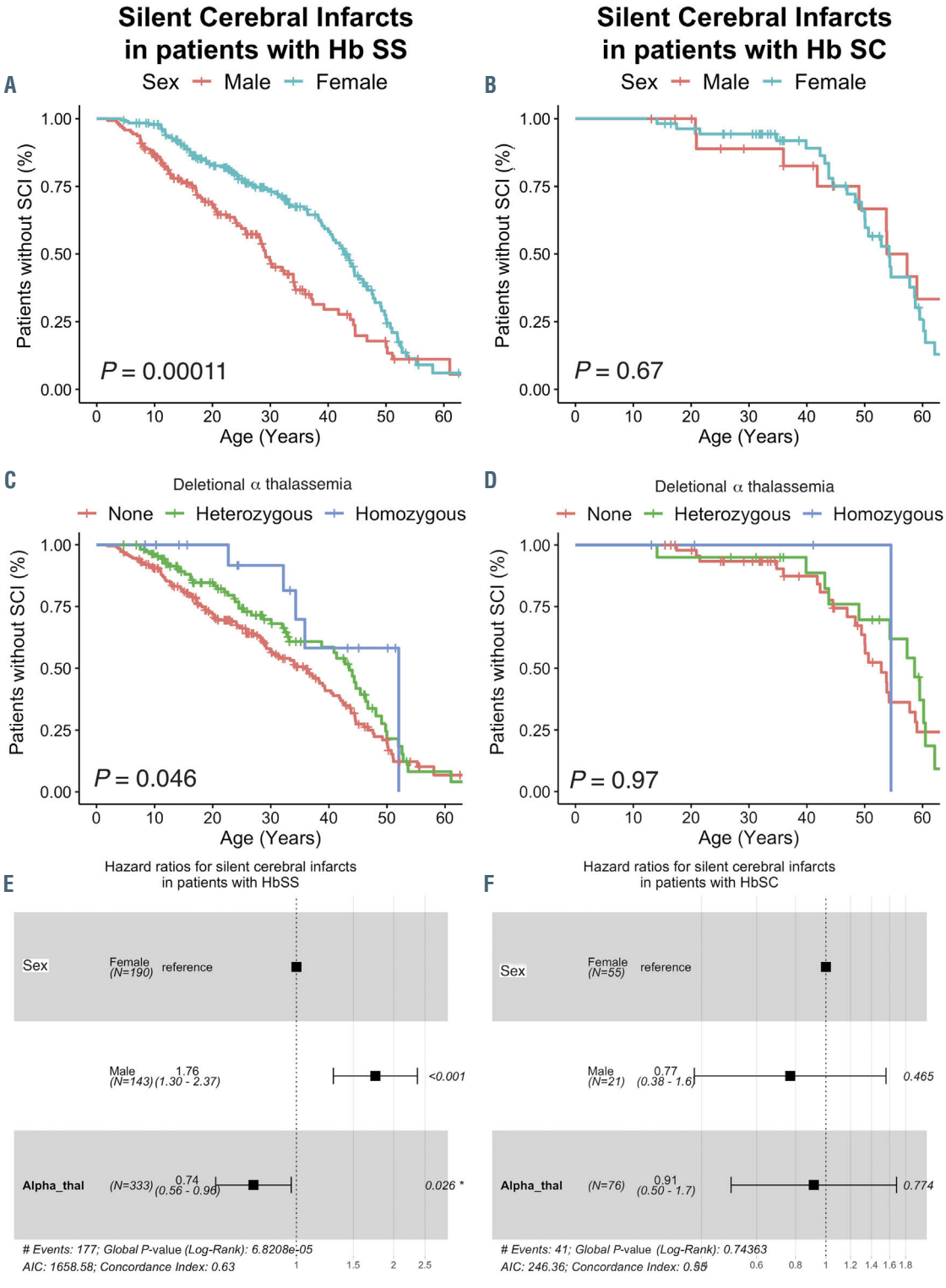

Figure 2. Survival analysis of factors effecting silent cerebral infarcts events in patients with HbSS and HbSC disease separately. (A) Kaplan-meier plot comparing outcomes in males and females in hemoglobin (Hb) SS. (B) Kaplan-meier plot comparing outcomes in males and females in HbSC. (C) Kaplan-meier plot comparing outcomes with no α thalassaemia (AT), heterozygous and homozygous of deletional α in patients with HbSS. D) Kaplan-meier plot comparing outcomes with no AT, heterozygous and homozygous of deletional α in patients with HbSS. (E) Forest plot of the Cox-proportionate hazard ratios of the factors affecting silent cerebral infarcts (SCI) outcomes in patients with HbSS. (F) Forest plot of the Cox-proportionate hazard ratios of the factors affecting silent cerebral infarcts outcomes in patients with HbSC.

effect (<76 g/L *vs.* >86 g/L).[3] However, there may also be an additional rheological benefit in the form of improved red blood cell deformability and reduced hemolysis reducing microinfarcts. Unfortunately, we did not have sufficient data on baseline Hb levels in this cohort to assess the interaction of AT and Hb on SCI.

Our study had some important negative findings. Some studies have found low HbF levels to be a risk factor[4-6] for SCI, 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 SCI.[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 AT and female sex, but not elevated HbF%, provide protection against development of SCI in patients with HbSS. SCI 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.

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