HHS Public Access

Author manuscript

Br J Haematol. Author manuscript; available in PMC 2019 January 01.

Published in final edited form as:

Br J Haematol. 2018 January ; 180(1): 134–146. doi:10.1111/bjh.15011.

Clinical and genetic factors are associated with pain and hospitalisation rates in sickle cell anaemia in Cameroon

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Summary

We aimed to investigate the clinical and genetic predictors of painful vaso-occlusive crises (VOC) in sickle cell disease (SCD) in Cameroon. Socio-demographics, clinical variables/events and haematological indices were acquired. Genotyping was performed for 40 variants in 17 pain-related genes, three fetal haemoglobin (HbF)-promoting loci, two kidney dysfunctions-related genes, and *HBA1*/*HBA2* genes. Statistical models using regression frameworks were performed in R®. A total of 436 hydroxycarbamide- and opioid-naïve patients were studied; median age was 16 years. Female sex, body mass index, Hb/HbF, blood transfusions, leucocytosis and consultation or hospitalisation rates significantly correlated with VOC. Three pain-related genes variants correlated with VOC (*CACNA2D3*-rs6777055, p = 0 .025; *DRD2*-rs4274224, p = 0 .037; *KCNS1*-rs734784, p = 0.01). Five pain-related genes variants correlated with hospitalisation/consultation rates. (*COMT*-rs6269, p = 0.027; *FAAH*-rs4141964, p = 0.003; *OPRM1*-rs1799971, p = 0.031; *ADRB2-rs*1042713; p < 0.001; *UGT2B7*-rs7438135, p = 0.037). The 3.7 kb *HBA1/HBA2* deletion correlated with increased VOC (p = 0.002). HbF-promoting loci variants correlated with decreased hospitalisation (*BCL11A*-rs4671393, p = 0.026; *HBS1L-MYB*-rs28384513, *p* = 0.01). *APOL1* G1/G2 correlated with increased hospitalisation (p = 0.048). This first study from Africa has provided evidence supporting possible development of genetic risk model for pain in SCD.

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Authorship Contributions: Conceived and designed the experiments: A.W, K.M., C.D. Performed the experiments: K.M., V.J.N.B. Patients recruitment, sample and clinical data collection and processing: V.J.N.B., B.C.C. Data analysis: K.M., A.P.K., E.C., A.W. Contributed reagents/materials/analytic tools: V.J.N.B., B.C.C., C.D., A.P.K., AW. Wrote the paper: A.W K.M., A.W. Revised and approved the manuscript: K.M., V.J.N.B., B.C.C., C.D., E.C., A.P.K.

Conflict of Interest Disclosures: The authors report no conflicts of interest, and take full responsibility for the content and writing of this article.

Keywords

Sickle cell disease; Acute vaso-occlusive painful crises; Genetics; Cameroon; Africa

Introduction

Acute episodes of pain or vaso-occlusive crises (VOC) are hallmarks of sickle cell disease (SCD). Frequent VOC were a marker for disease severity and premature mortality in the Cooperative Study of Sickle Cell Disease (CSSCD) (Platt *et al*, 1991; Platt *et al*, 1994), and in modern cohorts in the United States of America (USA) (Darbari *et al,* 2013; Elmariah *et al*, 2014). VOC have a major economic impact due to the cost of unscheduled health care, and mostly affect the coping ability of SCD patients (Wonkam *et al*, 2014a; Kanter and Kruse-Jarres 2013). The pathophysiology of vaso-occlusion involves multiple interrelated processes that have been increasingly linked to inflammation (Owusu-Ansah *et al*, 2016). Erythrocyte sickling and haemolysis trigger acute inflammation, marked by elaboration of inflammatory cytokines which stimulate nociceptors on peripheral nerve endings (Ballas *et al*, 2012) and abnormal expression of endothelial adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM1), E-selectin and P-selectin, that are now targets of new therapies for VOCs in SCD (Hoppe *et al*, 2017; Ataga *et al*, 2017). There are inter-individual variations in frequency and severity of VOC, leading to differential utilization of acute care. In the CSSCD, SCD patients with three to 10 VOC episodes a year represented only 5.2 % of the sample, yet accounted for 32.9 % of VOC episodes (Platt *et al*, 1991). Similar data were also recently reported despite availability of modern SCD-specific therapies (Darbari *et al*, 2013). Higher haematocrit and lower fetal haemoglobin (HbF) are strong predictors of frequent VOC (Platt *et al*, 1991), and are subject to genetic modifiers.

Genetic variants at three principal loci, including *BCL11A, HBS1L–MYB* and *HBB* cluster, account for 10–20% variations in HbF levels among SCD patients in the USA and Cameroon (Lettre *et al*, 2008; Wonkam *et al*, 2014b), and these variants have been associated with VOC in SCD (Lettre *et al*, 2008; Sheehan *et al*, 2013). Co-inheritance of α-thalassaemia has been inconsistently associated to variable levels of VOC (Platt *et al*, 1991; Darbari *et al*, 2012; Tarer *et al*, 2006). In addition, a few observational studies have explored the associations of VOC with targeted variants in genes coding for enzymes that metabolize analgesics or inflammation-related proteins, with encouraging results (Hu *et al*, 2016; Jhun *et al*, 2015; Mendonça *et al*, 2010; Belfer *et al*, 2014; Galarneau *et al*, 2013). Specifically, one study identified and prioritised a total of 115 single nucleotide polymorphisms (SNPs) in 49 candidate genes that modified pain among African-American SCD patients (Jhun *et al*, 2015); but this has not been followed by genotype to phenotype investigations. We are aware of a related study conducted in Africa where nearly 80% of new SCD patients are born (Piel *et al*, 2013).

Cameroon is a sub-Saharan African country with approximately 20 million people. The frequency of sickle cell mutation ranges from 8 to 34% in Cameroon (Weatherall and Clegg, 2001). There is currently no provision of universal new-born screening for SCD in the country and the median age of SCD diagnosis is 3.3 years (Wonkam *et al*, 2014b). There are

no specialized centres for lifelong medical treatment, resulting in very few patients being exposed to hydroxycarbamide or opioid treatment (Wonkam *et al*, 2014a).

The primary objective of the present study was to investigate targeted genetic variants associated to VOC episodes in a group of patients living with SCD in Cameroon. The secondary objective was to study the association of these variants with health care utilisation (hospitalisations or consultations), considered as direct proxies of VOC.

We have investigated 23 targeted variants in 17 pain-related genes and the correlation of VOC with established genetic modifiers of SCD, namely, the 3.7 *HBA1/HBA2* deletion, variants in HbF-promoting loci, and kidney dysfunction-associated variants *(APOL1* and *HMOX1*), which have been correlated with SCD nephropathy in Cameroon (Geard *et al*, 2017).

Materials and Methods

Ethical approval

The study was approved by the University of Cape Town, Faculty of Health Sciences Human Research Ethics Committee (HREC REF: 661/2015), Cape Town, South Africa; and the National Ethics Committee of the Ministry of Public Health, Yaoundé, Republic of Cameroon (No. 033/CNE/DNM/07). All patients older than 18 years signed consent forms, while informed consent was given by the parents or guardians for participants younger than 18 years old, in accordance with the declaration of Helsinki.

Patients

Assessment of clinical events—Patients were prospectively recruited at the Yaoundé Central Hospital and Laquintinie Hospital in Douala, between January 2010 and December 2011. Socio-demographic and clinical events were collected by means of a structured questionnaire administered to parents/guardians and adult SCD patients. Patients' medical records were reviewed, to delineate their clinical features over the past three years. Specifically, the occurrence of VOC, consultations rates referring to outpatient visits, hospitalisation rates, and blood transfusion history. Painful VOC events were defined as the occurrence of pain in the extremities, back, abdomen, chest or head that lasted at least two hours, and that could not be attributed to causes other than SCD, and required a hospital visit, and treatment with non-opioid antalgics (Platt *et al*, 1991). Body mass index (BMI) and blood pressures (BP) were measured in the outpatient settings.

Only patients older than five years of age (to avoid age-related changes in the complete blood count and HbF level), who had not received a blood transfusion or hospitalisation in the past 6 weeks were included. None was currently treated with hydroxycarbamide or opioids.

Control participants—For the purpose of comparative allele frequencies of targeted variants in selected pain-related genes of interest, a total of 105 ethnically matched Cameroonian controls (HbAS and HbAA) were randomly recruited, from apparently healthy blood donors in Yaoundéfor participation in the study.

Measurements of haematological indices and renal functions—Routine blood counts of patients and haemoglobin (Hb) electrophoresis were conducted on arrival at the hospital, at the haematological laboratory of the Centre Pasteur in Yaoundé, as previously described (Wonkam *et al*, 2014b). Routine laboratory tests were performed to measure serum creatinine. The urine albumin level was determined using either the Siemens Clinitek Status test (Erlangen®, Germany) or the Hemocue Albumin 20 system (Angelholm®, Sweden) as describe elsewhere (Geard *et al*, 2017). The glomerular filtration rate (GFR) was estimated (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (Geard *et al*, 2017).

Molecular methods

Sickle cell anaemia mutation, *HBB* cluster haplotypes, and 3.7 kb *HBA1/HBA2* deletion—DNA was extracted from peripheral blood following the manufacturer's instructions (Puregene Blood Kit; Qiagen, Hilden, Germany). Molecular analysis to determine the presence of the sickle mutation was carried out on 200 ng DNA by polymerase chain reaction (PCR) to amplify a 770 bp segment of the *HBB*, followed by DdeI restriction analysis of the PCR product (Saiki *et al*, 1985). The present analysis was restricted to sickle cell anaemia (homozygous HbS) due to the well-known differences in laboratory parameters (Platt *et al*, 1991; Darbari *et al*, 2013), and to allow single sickle genotype (HbSS) for genetic associations. Using published primers and methods, five restriction fragment length polymorphism (RFLP) sites in the *HBB* cluster were amplified to analyse the XmnI ($5'^{G}\gamma$), HindIII ($^{G}\gamma$), HindIII ($^{A}\gamma$), HincII ($3\psi\beta'$) and HinfI ($5'\beta$) for the *HBB* haplotype background (Bitoungui *et al*, 2015). The 3.7 kb *HBA1/HBA2* deletion was successfully screened, using the expand-long template PCR (Roche Diagnostics, Basel, Switzerland), as previously published (Rumaney *et al*, 2014).

SNPs in HbF-promoting loci, *APOL1* and *HMOX1*—Ten regions containing specific SNPs were amplified: viz, for the *BCL11A* locus, SNPs rs11886868 and rs4671393; for the *HMIP1/2* loci: SNPs rs28384513, rs9376090, rs9399137, rs9389269; rs9402686 and rs9494142; for the *OR51B5/6 loci*: SNP rs5006884, for *HBG2* loci, SNP rs7482144; followed by Sanger sequencing (Wonkam *et al*, 2014b). SNP genotyping of rs60910145 (*APOL1*), rs73885319 (*APOL1*) and rs743811 (*HMOX1*) was performed using predesigned TaqMan genotyping assays (Applied Biosystems, Foster City, CA, USA), and the genotyping of rs3074372 (*HMOX1*) and rs71785313 (*APOL1*) variants using fragment analysis, incorporating fluorescently-labelled forward primers (Geard *et al*, 2017).

Genotyping of targeted SNPs in pain-related genes

Selection of SNPs: We initially performed a thorough review of the literature on pharmacogenomics of SCD therapeutics, and identified a list of variants that are potentially associated with pain in SCD (Mnika *et al*. 2016). Once the SNPs of interests were identified, we investigated their allele frequencies in African populations present in the 1000 Genomes project (http://www.internationalgenome.org/home), and further narrowed the selection to SNPs that showed high frequency among African populations. For the purpose of additional quality control *ADRA2A*-rs3750635, which was monomorphic for all the populations in the

1000 Genomes project, was also genotyped. This resulted in the selection of 23 SNPs from 17 pain-related genes that were investigated in the present study (Table I).

Genotyping: SNPs were genotyped using a TaqMan® SNP Genotyping Assay and TaqMan® Universal Master Mix (Life Technologies, Carlsbad, CA, USA), at the Division of Human Genetics, Faculty of Health Sciences, University of Cape Town; and by iPLEX GoldSequenom Mass Genotyping Array (Inqaba Biotec, Pretoria, South Africa). Validation was done in a subset of sample (10%), by Sanger sequencing using BigDye terminator mix (Promega, Madison, WI, USA).

Statistical Analysis

Descriptive statistics was performed using STATA, version 14.0.370 (StataCorp, College Station, TX, USA). For quality control, a Hardy-Weinberg Equilibrium (HWE) test was performed on all genotype results. Two SNPs were monomorphic in both patients and controls: *HBS1L-MYB*-rs9376090 and *ADRA2A*-rs3750635; *OPRM1*-rs1799971 was monomorphic among controls and very rare (0.001) in patients. Only the 3.7del α-globin gene genotypes ($p = 0.005$) were out of HWE; however, this deviation was expected in view of the strong protective effect of this genetic variant on SCD, as previously reported on Cameroonians (Geard *et al*, 2017, Rumaney *et al*, 2014). The skewness of VOC, and hospitalisation, consultation rates and haematological indices, was corrected by taking their natural logarithm to approximate normal distribution. Prior to log transformation, these variables were all rescaled by systematically adding a constant (one) to allow the inclusion of participants with null values. General linear and multinomial regression frameworks, adjusted for age and sex, were performed to investigate the relationship between genotypes results and clinical data, using the R® statistical software (version 3.3.3, The R Foundation for Statistical Computing, Vienna, Austria). *P*-values < 0.05 were considered statistically significant. For association analysis with pain-related genes, and modifiers of sub-phenotypes of SCD, the Bonferroni critical p-value is also provided to indicated the threshold for significance after accounting for multiple comparisons.

Results

Description of the studied cohort

A total of 436 SCD patients (HbSS) were included; Table II summarizes the participants' characteristics. There was roughly equal numbers of males and females (217 and 219, respectively). The median age was 16 years. The most prevalent β-globin like gene cluster haplotypes was Benin, followed by Cameroon. Up to 41.8% ($n = 151$) of patients had co-inherited a single or double 3.7 kb *HBA1/HBA2* deletion. The median number of VOC per year was 2 (range: 0-40); 46.6 % ($n = 185$) of participants had ≥ 3 VOC per year and 27.2 % ($n = 115$) had ≥ 2 hospitalisations per year.

Clinical and haematological factors associated with acute pain crisis

Several clinical factors significantly correlated with the number of VOC (Figure 1 and Figure S1), including female sex (estimate = 0.073; p = 0.026), hospitalisation rates (estimate = 0.41, p = < 0.0001), consultation rates (estimate = 0.254, p = <0.0001), BMI

(estimate = 0.022; p = 0.02.) and positive history of blood transfusion (estimate = 0.42; p = 0.046; Figure S1).

The number of VOC was also associated with various haematological indices, including Hb level (estimate = -0.074; p = 0.005), white blood cell counts (estimate = 0.008; p = 0.04) (Figure 1); red blood cell counts (estimate = -0.154; p = 0.015; figure S1) and HbF level (estimate = -0.003; p = 0.025; Figure S1). There was no significant association between VOC and age (estimate = -0.003, p = 0.19), microalbuminuria (estimate = 0.050; p = 0.198), platelet count (estimate = -2.437e-05; p = 0.927), eGFR (estimate = -0.0001; p = 0.942), systolic BP (estimate = 0.0009; p = 0.794), and diastolic BP (estimate = 0.0009; p = 0.851).

Frequencies of pain-related genes variants across various populations

The differential frequencies across populations of the SNPs investigated are presented in Tables I and S1. When excluding the monomorphic pain-related genes SNPs, a total of 6/21 SNPs (28.6%) were differentially distributed among Cameroonian SCD individuals compared to controls (Table I); all but one (5/6) showed significant or borderline association with VOC or hospital utilisation (Table I). Up to 40.1% of the variants studied (9/22) were differentially frequent when comparing Cameroonian vs. African American patients living with SCD. Furthermore, comparison with control data extracted from the 1000 Genome Project, showed significant differences in allele frequencies in half of SNPs with Africans (11/22), and for the large majority of SNPs (88.8%; 18/22), with both Europeans and Asians (Table S1).

Correlations of VOC, health care utilisation and pain-related genes variants

Three pain-related gene variants significantly correlated with VOC (*CACNA2D3*-rs6777055, p = 0.025; *DRD2*-rs4274224, p = 0.037; and *KCNS1*-rs734784, p = 0.01); all these three variants, were significantly or borderline associated with hospitalisation rates (Table III; Figure 2). SNPs in four genes were borderline associated with VOC (*ABCB1*-rs1045642, p = 0.065; *AVPR1A*-rs10877969; p = 0.072; *FAAH*-rs4141964, p = 0.084; *TRPA1*-rs920829, p = 0.078); two of which were also significantly or borderline associated with hospitalisation rates (Table III).

Five pain-related genes variants correlated with hospitalisation or consultation rates, without any significant association with VOC (Table III; *COMT*-rs6269, p = 0.027; *FAAH*-rs4141964, p = 0.003; *OPRM1*-rs1799971, p = 0.031; *ADRB2* -rs1042713; p < 0.001; and *UGT2B7*-rs7438135, p = 0.037).

Correlations of VOC, hospitalisation rates and variants in established genetic modifiers of SCD

The 3.7 kb *HBA1/HBA2* deletion correlated with increased VOC (p = 0.002) and related hospitalisation rates (p = 0.02) (Table IV, Figure 3A). Variants in all the HbF-promoting loci correlated mostly with decreased hospitalisation rates (*BCL11A-rs*4671393, p = 0.026; *HBS1L-MYB*-rs28384513, *p* = 0.01; and *HBS1L-MYB*-rs9494142, p = 0.038; Figure 3B, C); but *BCL11A-rs*4671393 was also associated with decreased VOC (p = 0.017). *APOL1*

G1/G2 correlated with increased hospitalisation rates (p = 0.048, Table IV, Figure 3D). Variants in *HMOX1* were not associated with VOC or hospitalisation rates (Table 4IV).

Discussion

To our knowledge, this is the first study to investigate targeted genomic variants in relation with VOC in SCD in Africa, where the burden of SCD is very high, with a mostly non-advantageous environment for SCD patients. Hydroxycarbamide and opioid medications that are widely used in high income settings, are serious pharmacological modifiers of both pain crisis and health care utilization, and therefore are intrinsic limitations of similar observational studies (Darbari *et al*, 2013). In Cameroon, the non-advantageous physical environment, characterised by high temperature (which could trigger dehydration and VOC) and the endemicity of malaria (which often deteriorates the anaemia in SCD), superimposes on ill-health systems that have a lack of newborn screening and specialised centres for SCD, and lack of hydroxycarbamide or anti-bioprophylaxis. In addition, there is no universal medical insurance coverage in Cameroon, and care of SCD patients therefore relies on out-of-pocket spending by family members. However, poverty in Cameroon affects more than 50% of the rural population and up to 30% of the urban population (World Health Organization 2010), which in turn means that the financial burden of the necessary medical care for SCD often cannot be met (Wonkam *et al*, 2014a). Consequently, patients in Cameroon frequently suffer exceptionally severe SCD sequelae, such as stroke (Njamnshi *et al*, 2006), and chronic kidney dysfunctions (Geard *et al*, 2017). This could mean that many SCD patients are unlikely to survive beyond childhood, unless possibly subjected to positive selective pressure of various genetic modifiers. Therefore, this particularity deleterious environment in Africa could offer a unique opportunity to reveal important genetic modifiers in SCD patients, which make the findings described in the present paper even more important, and will warrant urgent replications in other African settings. This article is also unique because of the large number of clinical variables and selected SNPs in both specific pain-related genes and established modifiers included. It is therefore reasonable to envisage expending future explorations with genome-wide association studies, and targeted deep sequencing of genes in the inflammatory pathways, in SCD patients living in Africa. Nearly half of the participants (46.6 %) had frequent VOC episodes, much higher than the 5.2% reported in the CSSCD (Platt *et al*, 1991), revealing a particular severity of this disease in Cameroon (Wonkam *et al*, 2014a; Njamnshi *et al*, 2006; Geard *et al*, 2017). But this higher rate of pain could also be attributed to a selection bias, due to hospital-based recruitment, and the cross-sectional and retrospective nature of the VOC phenotype. Although the age dependency of VOC is well documented (Platt *et al*, 1991; McMillan *et al*, 2015), we did not find such an association, probably due to the much younger age of our cohort. We observed an increased number of VOC in females; sex-specific genetic susceptibility in pain-related genes has been reported in SCD (Belfer *et al*, 2014). We observed a positive correlation of VOC with BMI; whether this is related to the high-density lipoprotein cholesterol level, reported to be independently associated with VOC requiring hospitalisation (Darbari *et al,* 2013), remains to be investigated. Nevertheless, among African American patients with SCD (19% overweight/obese), BMI status did not influence the frequency or duration of hospitalisations for VOC (Zivot *et al*, 2017). As previously reported, SCD patients with

more severe anaemia are at risk for increased VOC (Platt *et al*, 1991; Darbari *et al*, 2013); this is additionally supported here by the significant association of VOC with history of blood transfusion (Figure S1). Worsening of anaemia is a frequently observed complication of acute painful VOC, which is often treated with red cell transfusions (Darbari et al. 2013), but some studies suggest that increased anaemia is associated with fewer VOC (Kato *et al*, 2017). In accordance with other studies, HbF reduces sickle haemoglobin polymerization, vaso-occlusion and hospitalisation in SCD in the USA (Charache *et al*, 1995; Lettre *et al*, 2008).

Variants in HbF-promoting loci have been associated with higher total haemoglobin concentrations and lower leucocyte counts (Sheehan *et al*, 2013; Mtatiro *et al*, 2014), as well as lower VOC and composite endpoints, such as hospitalisations (Lettre *et al*, 2008; Sheehan *et al*, 2013; Leonardo *et al*, 2016). Co-inheritance of α-thalassaemia is protective against some SCD-related complications, such as acute chest syndrome, leg ulcers and chronic kidney disease (Geard *et al*, 2017; Higgs *et al*, 1982; Guasch *et al*, 1999), but convey similar or higher rates of VOC (Meier et al, 2017; Platt *et al*, 1991; Darbari *et al*, 2013; Darbari *et al*, 2012; Tarer *et al*, 2006). In the present study, we have observed higher rates of VOC with the co-inheritance of α-thalassaemia (Figure 3). In the CSSCD, the slight increase in the pain rate associated with α-thalassaemia was attributable to the higher haematocrit (Platt *et al*, 1991). *APOL1* G1/G2 risk alleles were previously associated with kidney dysfunctions among Cameroonians living with SCD (Geard *et al*, 2017), and were associated with increased hospitalisation rates in the present study (Table IV). This could indicate that some hospitalisations were probably due to other confounding causes. There is evidence that acute kidney injury is common during sickle cell pain crisis (Baddam *et al*, 2017); but, we did not observe any association between VOC and albuminuria, eGFR or variants in *HMOX1;* although variants in *HMOX1* were previously associated with kidney dysfunctions among Cameroonians with SCD (Geard *et al*, 2017), and reduced acute chest syndrome and hospitalisation rates in SCD patients in the USA (Bean *et al*, 2012). Increased health care utilization by SCD patients for VOC is well known (McMillan *et al*, 2015; Rees *et al*, 2010), and these individuals are at particularly high risk for death (Platt *et al*, 1991, Darbari *et al*, 2013; Elmariah *et al*, 2014), and should be vigorously treated; early identification of these individuals could involve a comprehensive risk model including evolving variants specific in pain–related genes.

The majority of SNPs (5/6) in pain-related genes differentially frequent in Cameroonian SCD vs. control were borderline-to-significantly associated with VOC or healthcare utilizations, indicating possible selection and enrichment of protective variants among SCD patients (Table I), owing to the unfavourable environment. Thus, the allele frequencies reported in the studied patients may not be representative of the entire SCD population in Cameroon, as it is possible that these patients are less severe cases who have survived childhood. Allele frequencies were significantly different among patients from Cameroon vs. African American living with SCD for 40.1% of the SNPs (9/22 SNPs; Table I), and even more so with African data extracted from the 1000 Genomes Project (11/22, 50 %; Table S1). This is in line with the high level of genetic variations in populations of African ancestry (Gurdasani *et al*, 2015).

In total, variants in 8of the 22 (36.4%) pain-associated genes investigated were significantly associated with VOC or consultations/hospitalisation rates (Table III). These are novel findings. SNPs located in a subunit of the calcium channel gene *CACNA2D3*, were also associated with a higher risk of anaemia, suggesting that calcium channels could potentially be involved in pathways for iron uptake in physiological conditions and in SCD (Baeza-Richer *et al*, 2015). A genome wide meta-analysis showed *DRD2* (Dopamine D2 receptor) genetic variations in the modulation of systolic BP among African Americans with SCD. We also found *DRD2-rs*4274224 to be associated with *VOC* in SCD patients (Table III). In addition, exploratory findings have suggested that *DRD3*-rs6280 (Ser9Gly) may contribute to pain heterogeneity in SCD (Jhun *et al*, 2014). Also related to our findings, variants in *KCNS1* were associated with and multiple chronic pain states in a non-SCD population (Costigan *et al*, 2010). Five pain associated-genes variants correlated with health services utilization only (Table III). These include variants in *COMT* (catechol-O-methyltransferase), *OPRM1* (opioid receptor mu *1* gene), *UGT2B7 (*UDP glucuronosyltransferase family 2 member B7) and *ABCB1* (ATP binding cassette subfamily B member 1); all previously suggested as potentially important for SCD VOC (Joly *et al*, 2012; Darbari *et al*, 2008). Other studies have found that *COMT-rs*4680 (158 Met allele or Met/Met genotype) was associated with acute care utilization, an indicator of acute pain (Jhun *et al*, 2014). The presence of the *UGT2B7*-840G allele contributes to the variability in hepatic clearance of morphine in SCD (Eyler *et al*, 2008). Up to 35% of African American SCD patients were previously reported to have variants in *ABCB1,* suggested to potentially influence good morphine exposure (Joly *et al*, 2012; Darbari *et al*, 2008).

Limitations

Possible limitations of the present study are the cross-sectional nature and the hospital-based recruitment. VOC episodes may have been subjected to pain self-tolerance bias, and financial factors could have been also limiting factors for hospital attendance. The issue of chronic pain was difficult to address with the study design, as it seems highly likely that individuals with 40 pain events per year are experiencing chronic pain. However, self-reported VOC in SCD has also been useful as a clinical endpoint in drug trials, patient quality of life measures and as a prognostic marker for mortality (Platt *et al*, 1991, Charache *et al*, 1995; Keller *et al*, 2017; Machado *et al*, 2011; Hoots and Shurin, 2012). The possible poor definition of VOC is also tempered by its strong association with the use of health services, which was more objectively assessed; validating, to some extent, the use of such cost-effective patient reported outcomes for genetic association study. A recent genome-wide association study, which included only VOC episodes requiring hospitalisation, found that *KIAA1109*-rs3115229 approached genome-wide significance in a locus associated with auto-inflammatory disorders (Chaturvedi *et al*, 2017). Therefore, the present study represents an important step forward in understanding clinical and genetic predictors of VOC in sub-Saharan Africa and globally. Lastly, our findings must be interpreted while accounting for the possibility of chance findings in the context of multiple comparisons. Indeed, based on the Bonferroni corrected threshold p-value for significance, half of the pain-related gene variants associations with our outcomes of interest were borderline or non-significant. It is of note however that our study focused on previously characterized genes, and that changes remain: those associations could be significant at a corrected threshold p-

value in a bigger sample, while correction for multiple comparison always increases the chance of false negative findings.

Conclusion

This study has provided important findings on clinical predictors of acute painful episodes and the use of health care services in a unique group of SCD patients from Cameroon that have not been exposed to hydroxycarbamide and opioid. In addition, the study has identified specific variants of pain-related genes that are associated with acute pain crisis and health care utilization, as well as in established genetic modifiers of SCD, such as *HBA1/HBA2*, HbF- promoting loci and *APOL1*. Altogether, the results may improve our ability to identify SCD patients who are at elevated risk for VOC and other organ complications, and will contribute to refining the elaboration of risk-profiling strategies that integrate both genetic and clinical information. We acknowledge that implementation of any genetic risk model in SCD in Africa is clearly difficult today, due to multiple competing priorities: hopefully, as the costs of genomic tests decreases, it could be possible in future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The molecular experiments of the study were supported by grants from the National Health Laboratory Services (NHLS), South Africa; and the NIH, USA, grant number 1U01HG007459-01, and the Welcome Trust, Developing Excellence in Leadership, Training and Science (DELTAS) Africa, Award 107755Z/15/Z. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. K.M. is a PhD candidate at the University of Cape Town and this work is submitted in partial fulfilment of the requirement for their PhD.

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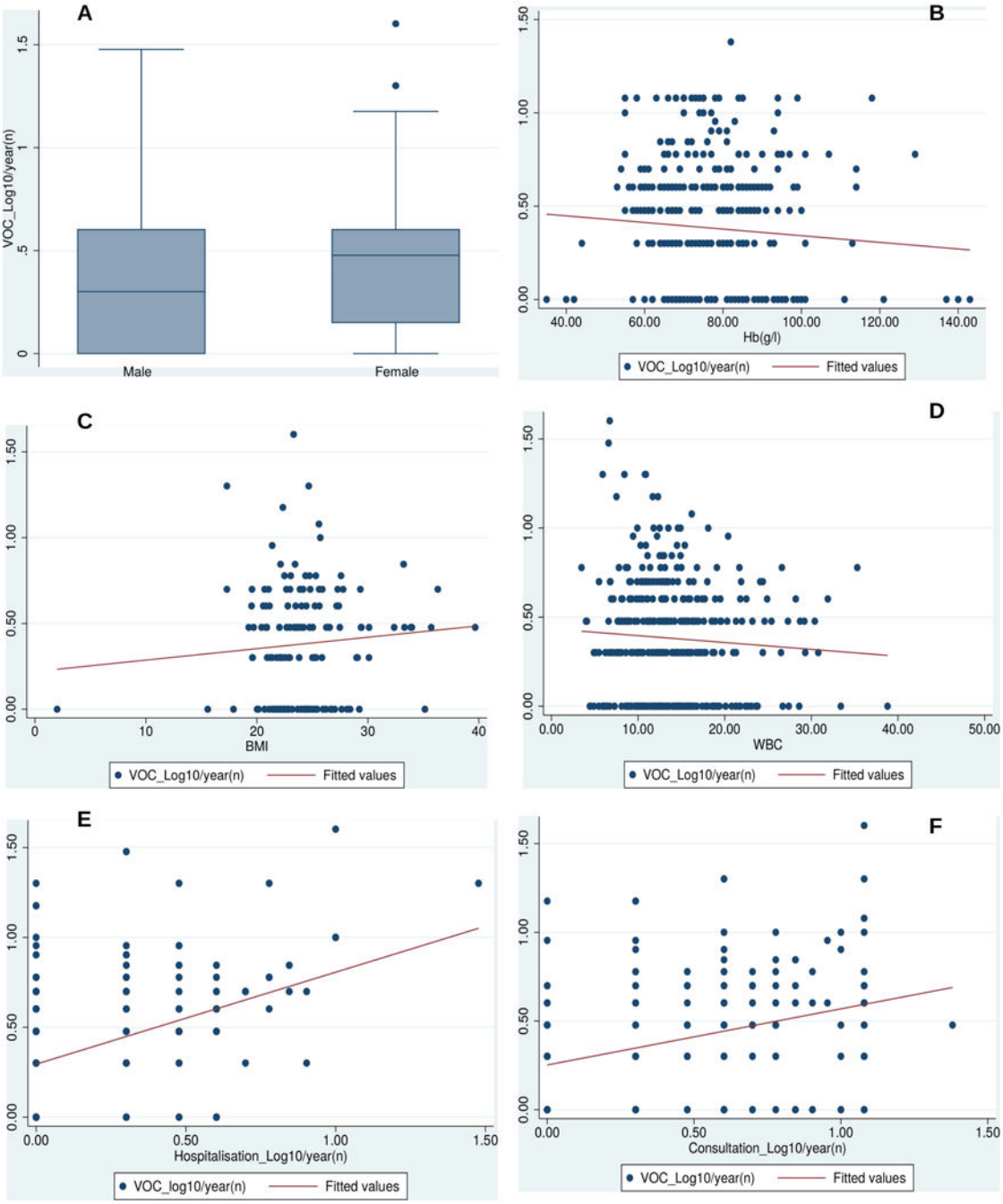

Figure 1. Scatter plot and box and whisker illustrating clinical and haematological factors associated with painful acute VOC episodes

(A) Box and whisker plots showing the correlation of vaso-occlusive crisis (VOC) values with gender (estimate = 0.073; p = 0.026). The horizontal lines that constitute the 'box' correspond to the lower quartile, median and upper quartile parameters. The length of the 'whiskers' that extend from the box in the upwards and downwards direction represent a distance 1.5 times the interquartile range. Values that lie outside this distance are considered outliers, or extreme values. (B) Clinical Factors associated with VOC in sickle cell disease with haematological indices. Scatter plot illustrating the negative relationship between log

VOC and total haemoglobin (Hb, g/l, estimate = -0.074; p = 0. 005); related to this was the association between VOC and red blood cell (RBC) counts (estimate = -0.0154; p = 0.015). (C) There was a positive correlation between body mass index (BMI) and VOC (estimate = 0.022; p = 0.02). (D) white blood cell (WBC) counts ($\times 10^9$/l) was also positively associated with VOC (estimate = 0.008; p = 0.04). Scatter plots illustrating the relationship between VOC values and log hospitalisations (estimated = 0.41; p = < 0.0001). The log hospitalisations variable is displayed on the x-axis, with the VOC values on the y-axis; the red line indicates the line of best fit. (F) Scatter plots illustrating the relationship between VOC values and log consultations (estimate = 0.254; p = < 0.0001); consultations variable is displayed on the x-axis, with the VOC values on the y-axis. The red line indicates a line of best fit.

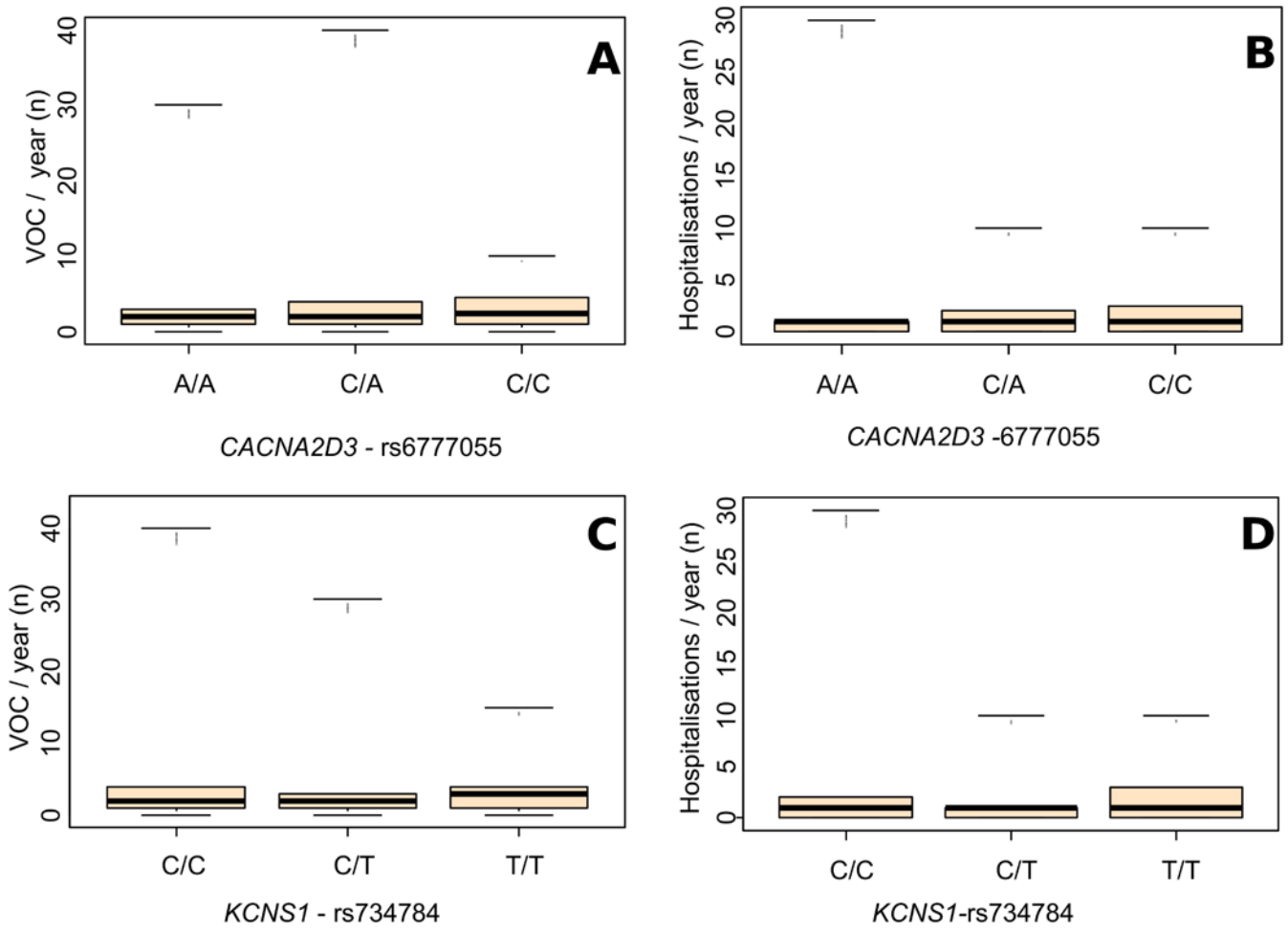

Figure 2. Associations between targeted variants in pain-related genes and painful acute VOC episodes or health services utilizations

(A) and (B) Box and whisker plots showing the association of *CACNA2D3-rs6777055* with vaso-occlusive crisis (VOC) ($p = 0.025$) and with hospitalisation rates ($p = 0.008$), respectively. (C) and (D). Box and whisker plots showing the association of *KCNS1-rs734784* with VOC ($p = 0.01$) and with hospitalisation rates ($p = 0.002$), respectively. Conventions are as per Figure 1.

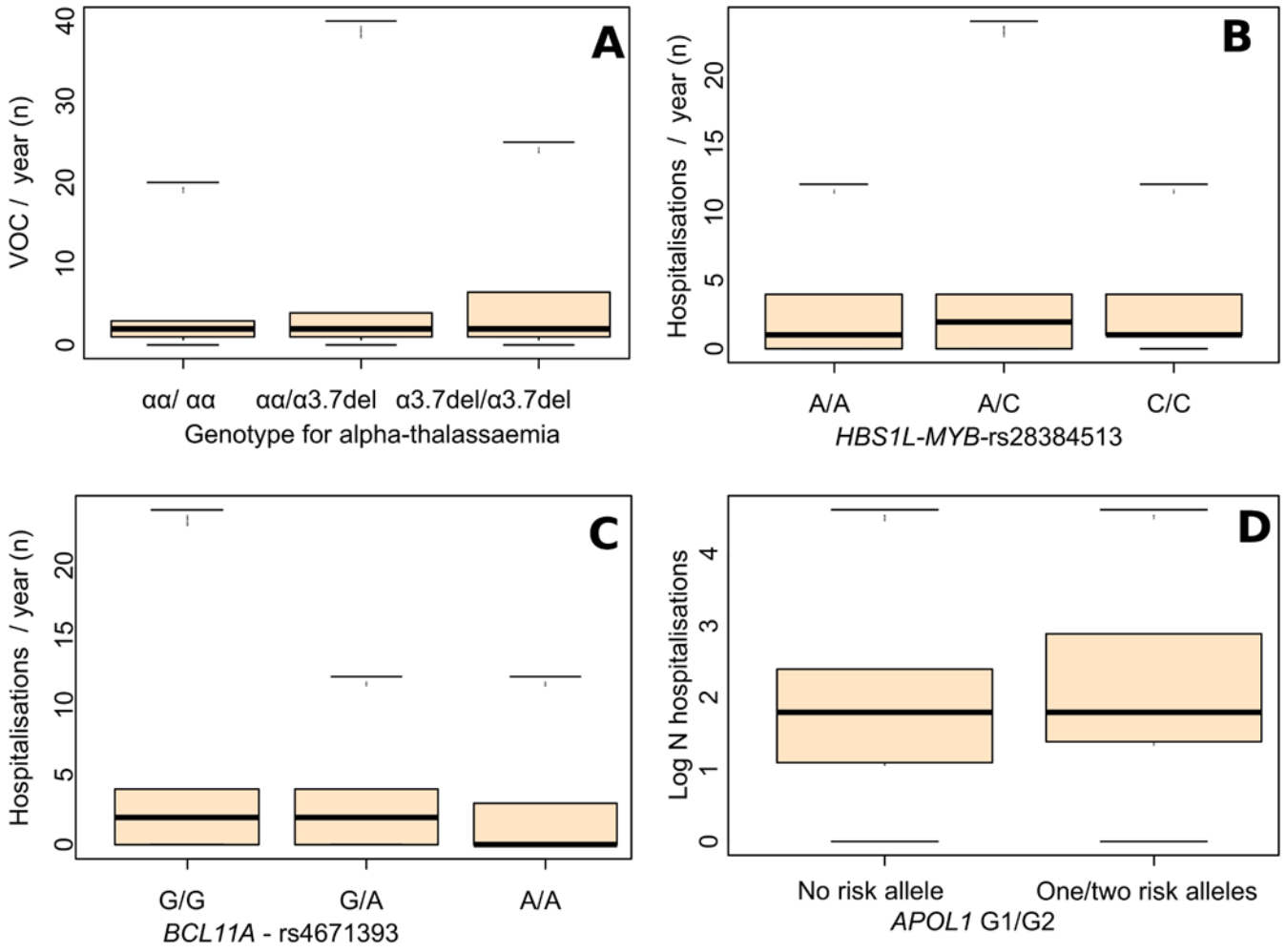

Figure 3. Associations between targeted variants in the alpha-globin gene, HbF-promoting loci and *APOL1*, with painful acute VOC episodes or hospitalisation rates

(A) Box and whisker plots showing the association of 3.7 alpha-globin gene deletions with vaso-occlusive crisis (VOC) (p = 0.002); a similar association was also reported with the hospitalisation rates (p = 0.02). (B) and (C) Box and whisker plots showing the associations of HbF- promoting loci variant with the hospitalisation rates: *HBS1L-MYB*-rs28384513 (p = 0.01); *BCL11A*-rs4671393 (p = 0.026); *BCL11A*-rs4671393 was also positively associated with VOC (p = 0.017), while *HBS1L-MYB*-rs28384513 was borderline with VOC (p = 0.057). (D) Box and whisker plots showing the association of *APOL1* G1/G2 risk alleles with hospitalisation rates (p = 0.048); *APOL1*-rs71785313 (G2) was borderline with the hospitalisation rates (p = 0.059). Conventions are as per Figure 1.

Table I

Allele frequencies of selected pain-related genes variants among Cameroonian and African American SCD cohorts

Gene	dbSNP ID	Position	Allele Change(s)	Cameroon Cohort		African American SCD*	Cameroon SCD vs Cameroon control P values	Cameroon SCD vs African American SCD P values
				SCD Cases	Controls			
ABCB1	rs1045642	87509329	T>C	0.153	0.205	0.785	0.076	**0.0001**
ADRA1A	rs1048101	26770511	T>C	0.177	0.162	0.776	0.607	0.286
ADRA2A	rs3750635	5750220	T>C	Monomorphic	Monomorphic	Monomorphic	NA	NA
ADRB2	rs1042713	148826877	A>G	0.481	0.5	0.514	0.615	0.409
ARRB2	rs1045280	4719343	C>T	0.342	0.135	0.558	**0.0001**	**0.0001**
AVPR1A	rs10877969	63153459	T>C	0.221	0.371	0.517	**0.0002**	0.305
BDKRB2	rs1799722	96204802	C>T	0.253	0.263	0.715	0.785	0.287
CACNA2D3	rs1851048	54587633	C>T	0.136	0.126	0.162	0.724	0.898
	rs6777055	55039890	A>C	0.195	0.2	0.803	0.863	**0.0001**
COMT	rs4633	19962712	C>T	0.239	0.283	0.620	**0.001**	**0.001**
	rs6269	19962429	A>G	0.44	0.421	0.672	0.634	**0.0008**
	rs4680	19963748	G>A	0.238	0.289	0.319	0.14	0.17
DRD2	rs4274224	113448730	C>T	0.262	0.263	0.290	0.977	0.501
FAAH	rs324419	46406314	T>C	0.128	0.208	0.154	**0.0035**	0.252
	rs2295632	46413890	T>G	0.249	0.31	0.711	0.079	0.485
	rs4141964	46399368	T>C	0.264	0.243	0.716	0.53	0.77
KCNS1	rs734784	45094986	A>G	0.469	0.439	0.548	0.445	0.591
OPRM1	rs1799971	154039662	A>G	0.001	Monomorphic	0.002	NA	0.575
STAT6	rs841718	57099213	C>T	0.317	0.365	0.701	0.191	**0.024**
	rs3024971	57099944	A>C	0.022	0.13	0.952	**0.0001**	0.666
TRPA1	rs920829	72065468	G>A	0.304	0.292	0.708	0.724	**0.0001**
TRPV1	rs222747	3589906	G>C	0.088	0.099	0.874	0.617	**0.0456**
UGT2B7	rs7438135	69095621	G>A	0.3	0.163	0.785	**0.0001**	**0.0001**

dbSNP ID; Single Nucleotide Polymorphism database identification; NA: not applicable; SCD: sickle cell disease. Significant P values are bolded.

*Jhun *et al* (2014); Jhun *et al* (2015)

Table II
Description of the studied Cameroonian SCD cohort

Variable		Median (25th- 75th percentiles) or %	Range	Observations (n)
Age (years)		16 (9-24)	5-54	436
Gender	Female/Male (219/216)		-	436
Haematological indices	RBC (x10^{12}/l)	2.7 (2.3-3.1)	1.4 -5.5	436
	Hb (g/l)	76 (67-85)	35-145	436
	MCV (fl)	84 (78-91)	59.0-117.0	436
	MCHC (g/l)	338 (316-358)	215-529	436
	WBC (× 10^9/l)	12.8(9.1-16.2)	2.9-49.8	436
	Lymphocytes (× 10^9/l)	5.2 (4.0-7.2)	0.2-22.6	436
	Monocytes (× 10^9/l)	1.3 (0.9-1.8)	011-7.8	436
	Platelet count (× 10^9/l)	374.3 (291.2-448.0)	97-756	436
	HbA2 (%)	3.6 (3.0-4.2)	0-18.2	436
	HbF (%)	8.8 (2.5-14.1)	0-37.4	436
Clinical events	VOC (n/year)	2 (1-4)	0-40	436
	Consultations (n/year)	2 (0-4)	0-24	324
	Hospitalisation (n/year)	1 (0-2)	0-30	422
	Blood transfusion (%)	77.8		330/424
	Stroke (%)	3.9		17/436
3.7 *HBA1/HBA2* deletion genotypes	αα / αα	59.8		225/376 [a]
	αα/ α3.7	30.1		113/376 [a]
	α 3.7/α 3.7	10.1		38/376 [a]
***HBB* Haplotype**	Benin/Benin	64.1%		212/331 [a]
	Benin/Cameroon	30.8%		102/331 [a]
	Cameroon/Cameroon	5.1%		17/331 [a]
Renal functions[£]	Crude albuminuria (mg/l)	41 (23-83)	3-1180	407
	eGFR (CKD-EPI) (ml/min/1.73m^2)	135.1 (112.0-154.4)	50.8-250.8	404
	Serum creatinine (μmol/l)	7 (5-8.5)	2-13.8	404

CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; eGFR: estimated glomerular filtration rate; Hb: haemoglobin; MCHC: mean corpuscular haemoglobin concentration; MCV: mean corpuscular volume; RBC: red blood cell count; SCD: sickle cell disease; VOC: vaso-occlusive crises; WBC: white blood cell count.

[a] Number of individuals, not alleles;

[£] previously reported in Geard ***et al*** (2017).

Table III

Variants in of selected pain-related genes and VOC, and consultation and hospitalisation rates

Gene	dbSNP ID	Position	Allele Change(s)	MAF	VOC P values	Effect Size (SE)	Consultations P values	Effect Size (SE)	Hospitalisations P values	Effect Size (SE)
ABCB1	rs1045642	87509329	T>C	0.153	**0.065***	0.152 (0.082)	0.741	0.206 (0.623)	0.417	0.386 (0.474)
ADRA1A	rs1048101	26770511	T>C	0.177	**0.094**	0.122 (0.058)	0.297	0.656 (0.627)	0.793	0.126 (0.477)
ADRA2A	rs3750635	5750220	T>C	Monomorphic	*NA*	NA	NA	NA	NA	NA
ADRB2	rs1042713	148826877	A>G	0.481	0.277	-0.328 (0.057)	**0.0004**$^{\partial}$	-0.143 (0.045)	0.25	-0.38 (0.329)
ARRB2	rs1045280	4719343	C>T	0.342	0.958	-0.0033 (0.063)	0.875	-0.143 (0.0474)	0.201	-0.464 (0.360)
AVPR1A	rs10877969	63153459	T>C	0.221	0.072*	0.101 (0.056)	0.119	0.672 (0.428)	**0.061***	-0.168 (0.066)
BDKRB2	rs1799722	96204802	C>T	0.253	0.64	0.034 (0.072)	0.591	-0.289 (0.537)	0.399	0.344 (0.406)
CACNA2D3	rs1851048	54587633	C>T	0.136	0.945	-0.006 (0.086)	0.633	-0.315 (0.658)	0.314	0.505 (0.499)
	rs6777055	55039890	A>C	0.195	**0.025***	-0.167 (0.074)	0.964	-0.0253 (0.562)	**0.008**	-0.181 (0.068)
COMT	rs4633	19962712	C>T	0.239	0.732	0.0345 (0.100)	0.122	1.185 (0.762)	0.225	-0.707 (0.580)
	rs6269	19962429	A>G	0.44	0.575	-0.0367 (0.065)	0.788	-0.134 (0.494)	**0.027***	0.138 (0.042)
	rs4680	19963748	G>A	0.238	0.986	0.0018 (0.102)	0.48	-0.553 (0.780)	0.264	0.667 (0.594)
DRD2	rs4274224	1.13E+08	C>T	0.262	**0.037**	0.148 (0.070)	0.078	0.955 (0.537)	**0.091***	0.695 (0.408)
FAAH	rs324419	46406314	T>C	0.128	0.663	-0.557 (0.227)	**0.076**$^{\partial}$	1.245 (0.908)	0.999	0.0012 (0.692)
	rs2295632	46413890	T>G	0.249	0.916	-0.0098 (0.093)	0.295	-0.733 (0.698)	0.653	0.239 (0.530)
	rs4141964	46399368	T>C	0.264	**0.084**$^{\partial}$	0.221 (0.122)	**0.058***	0.165 (0.086)	**0.003***	-185 (0.042)
KCNS1	rs734784	45094986	A>G	0.469	**0.010**$^{\delta}$	-0.165 (0.045)	0.581	0.265 (0.479)	**0.002**$^{\partial}$	0.229 (0.074)
OPRM1	rs1799971	1.54E+08	A>G	0.001	0.64	0.299 (0.53)	0.205	-1.047 (0.040)	**0.031**$^{\delta}$	-0.135 (0.044)
STAT6	rs841718	57099213	C>T	0.317	0.79	0.0176 (0.066)	0.358	0.465 (0.504)	0.959	-0.02 (0.381)
	rs3024971	57099944	A>C	0.022	0.262	0.213 (0.190)	0.35	-1.347 (1.42)	0.4	0.924 (1.10)
TRPA1	rs920829	72065468	G>A	0.304	**0.078**$^{\delta}$	-0.115 (0.050)	0.91	-0.0549 (0.485)	0.312	0.373 (0.368)
TRPV1	rs222747	3589906	G>C	0.088	0.907	0.0132 (0.112)	0.4	0.722 (0.856)	0.695	0.255 (0.650)
UGT2B7	rs7438135	69095621	G>A	0.3	0.805	0.028 (0.062)	**0.037**$^{\partial}$	-0.685 (0.043)	0.209	-0.459 (0.363)

MAF: minor allele frequency; NA: not applicable; SE: standard error; VOC: vaso-occlusive painful crisis rate. Significant / borderline P values are bolded:

= Codominant model,

* = Dominant model,

θ = Recessive model,

δ = Over dominant model; *NA* = not applicable; Bonferroni critical p-value <0.003.

Table IV

Variants in known modifiers of sub-phenotypes of SCD and VOC, and hospitalisation rates

Gene	dbSNP ID	Position	Allele Change(s)	MAF	VOC P values	Effect Size (SE)	Hospitalisations P values	Effect Size (SE)
HBA (3.7 Alpha-globin gene deletion)	16	NA	*NA*	**0.002**^{0}	0.339 (0.116)	**0.02***	0.17 (0.073)	
APOL1	rs60910145 (G1)	22:36265988	T>G	0.14	0.439	-0.061 (0.071	0.553	-0.075 (0.126)
APOL1	rs73885319 (G1)	22:36265860	T>G	0.13	0.67	-0.034 (0.067)	0.563	-0.070 (0.120)
APOL1	rs71785313 (G2) Indel	22:36266000-36266005	Deletion	0.082	0.784	0.102 (0.400)	0.059*	0.273 (0.1551)
APOL1	G1/G2	*NA*	NA	*NA*	0.194	-0.434 (0.114)	**0.048**$^{\delta}$	0.339 (0.200)
HMOX1	rs3074372	22:35380894	L>S	0.111	0.756	0.03 (0.084)	0.477	-0.509 (0.346)
	rs743811	22:35396981	T>C	0.111	0.234	-0.010 (0.063)	0.463	0.429 (0.061)
BCL11A	rs11886868	2:60493111	G>A	0.31	**0.081**^{0}	-0.20 (0.037)	**0.042***	-0.155 (0.171)
BCL11A	rs4671393	2:60493816	T>C	0.3	**0.017**^{0}	-0.334 (0.133)	**0.026***	-0.226 (0.087)
HBS1L-MYB	rs28384513	1.35E+08	A>C	0.217	0.057*	0.136 (0.058)	**0.010**$^{\delta}$	0.139 (0.045)
HBS1L-MYB	rs9376090	6:135090090	T>C	0.146	0.403	0.537 (0.033)	0.658	0.447 (0.062)
HBS1L-MYB	rs9399137	6:135097880	T>C	0.043	0.372	0.560 (0.033)	0.744	0.063 (0.100)
HBS1L-MYB	rs9389269	6:135106021	T>C	0.18	0.548	0.043 (0.060)	0.898	0.014 (0.10)
HBS1L-MYB	rs9402686	6:135427817	G>A	0.03	0.355	0.06 (0.11)	0.304	0.0.33 (0.058)
HBS1L-MYB	rs949414 2	6:135431640	T>C	0.11	0.343	-0.08 (0.076)	**0.038***	-0.163 (0.577)
HBG2	rs7482144	11:5254939	G>A	0.005	0.715	0.126 (0.404)	**0.008***	0.641 (0.184)
OR51B5/6	rs5006884	11:5352021	C>T	0.08	0.245	0.056 (0.032)	**0.056**^{0}	1.9 (0.057)

dbSNP ID; Single Nucleotide Polymorphism database identification; MAF: minor allele frequency; NA: not applicable; SCD: sickle cell disease; SE: standard error; VOC: vaso-occlusive painful crisis rate. Significant / borderline P values are bolded:

= Codominant model,

* = Dominant model,

^{0} = Recessive model,

$^{\delta}$ = Over dominant model; Bonferroni critical p-value <0.029.